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Preface

This volume of the *Methods in Molecular Biology* series is entirely devoted to the study of steroid receptor biology. Steroid hormone receptors represent a powerful system for the study of both the most fundamental molecular mechanisms of gene regulation and control and the gross physiological responses of organisms to steroid hormones. Research in this field has brought forth advances in the treatment of cancer, endocrine disorders, and reproductive biology, and allowed elucidation of the fundamental biological mechanisms of gene expression. In *Steroid Receptor Methods: Protocols and Assays*, the reader will find a collection of methods and protocols submitted by many fine steroid receptor researchers from throughout the world. These authors have been instructed to create a highly informative cross-section of the latest research techniques available. The resulting work is timely, useful, and approachable for both the experienced researcher and the novice to the field. Because the steroid receptor family is represented by a wonderfully diverse, yet strongly interrelated set of steroid receptor proteins, *Steroid Receptor Methods* contains protocols for the production and purification of a variety of receptor forms, including the progesterone, glucocorticoid, and androgen receptors. These procedures provide the raw material needed to conduct sophisticated biochemical analysis of receptor properties. Other techniques presented allow the reader to perform biochemical experiments on DNA binding characteristics, hormone binding assays, and protocols using combinatorial chemistry for drug discovery. Because steroid receptor effectiveness is influenced by a variety of cellular proteins, there are included in this volume a series of novel protocols utilizing the latest advances in immunochemistry, yeast two-hybrid screening, fluorescence, and other biochemical and cellular techniques to detect and detail these interactions. These techniques include both in vitro and in vivo approaches to provide the widest possible selection of tools to the modern biological researcher. Finally, in recognition of the growing importance of bioinformatics in biological research, several chapters have been included to guide and assist the modern research biologist in harnessing this increasingly valuable resource. These chapters locate and make accessible to the researcher the diverse computational tools currently available via the Internet. Taken together these chapters provide both novice and experienced researchers alike a set of invaluable tools to advance and extend their research.

Ben Lieberman, PhD

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I BIOINFORMATICS

1

Bioinformatics of Nuclear Receptors

Mark Danielsen

1. Introduction

Bioinformatics is a scientific discipline that is still being defined. To some, it is the development of new computer programs that use statistics to discern the relationships between DNA and protein sequences. To others, it is the development and implementation of databases to store and provide access to the sequences themselves and to related biological information. Finally, for still others, it is the use of the tools generated by computer scientists to analyze and interpret the information present in biological sequence data. Since a description of the bioinformatic tools available to study nuclear receptors could easily fill an entire book, this chapter first focuses on how to mine information on steroid receptors from databases on the World Wide Web (WWW), then gives a few examples of how to analyze the structure of a nuclear receptor using some of the available tools. A companion WWW site has been created for this chapter, where readers can find more detailed information and links (http://nrr.georgetown.edu/bioinform/begin.html).

2. Materials

The following subheadings list major WWW bioinformatics sites of interest to nuclear receptor aficionados (*see* **Note 1**).

2.1. Nuclear Receptor Resource (NRR) (http://nrr.georgetown.edu/nrr/nrr.html)

The NRR ***(1)*** is a collection of individual databases on members of the steroid and thyroid hormone receptor superfamily (*see* **Note 2**). Although the databases are located on different servers and are managed individually, they each form a node of the NRR. The NRR itself integrates the separate

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databases, and allows an interactive forum for the dissemination of information about the superfamily. It is therefore a particularly good site at which to start a search for information about nuclear receptors.

2.2. National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov)

The NCBI was established in 1988, under the auspices of the National Library of Medicine at the National Institutes of Health. The mission of the NCBI is to develop automated systems for the collection, storage, and retrieval of biological information, to gather information for these databases worldwide, to conduct research in bioinformatics, and to facilitate the use of databases and software developed at NCBI by the scientific community *(2)*.

2.2.1. GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html)

GenBank *(3)* is an annotated database of all publicly available DNA sequences. Sequences are collected as part of the International Nucleotide Sequence Database Collaboration, which is comprised of the DNA DataBank of Japan (DDBJ) *(4)*, the European Molecular Biology Laboratory (EMBL) *(5)*, and GenBank at NCBI. These three organizations exchange data on a daily basis. There are a number of divisions of GenBank that gather specific types of sequences, e.g., the Database of Expressed Sequence Tags, Database of Sequence Tagged Sites, and the Database of Genome Survey Sequences.

2.2.2. Entrez (http://www.ncbi.nlm.nih.gov/Entrez)

The NCBI has developed a search and retrieval system called "Entrez" that integrates individual databases hosted at NCBI. These databases include structural, taxonomic, nucleotide and protein sequence databases as well as literature and disease databases. The integrated nature of the Entrez system has made it perhaps the finest site for biological information retrieval on the WWW. What makes Entrez so useful is that most of the records are linked to other records, both within and between databases. Links within a database are called "neighbors" (e.g., Nucleotide Neighbors). Protein and nucleotide neighbors are determined by performing similarity searches, using the Basic Local Alignment Search Tool (BLAST) algorithm (*see* below) to compare the entry amino acid or DNA sequence to all other amino acid or DNA sequences in the database.

2.2.3. BLAST Sequence Searches (http://www.ncbi.nlm.nih.gov/BLAST)

The molecular databases at NCBI can be searched using BLAST *(6,7*; for a review, *see* **ref.** *8)*. A large array of databases can be searched, including: for nucleotides, GenBank, the Genome Sequence Database, and sequences

from the patent office; for proteins, Protein Information Resource (PIR), SWISS-PROT, Protein Research Foundation, Protein Data Bank (PDB) (sequences from solved structures), and translated coding regions from DNA sequences in GenBank. Two variations of BLAST, Position Specific Iterated BLAST (PSI-BLAST) and Pattern Hit Initiated BLAST (PHI-BLAST) can be used to search protein sequences ***(6)***.

2.2.4. Genomes Database (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Genome)

The Genomes database provides views for a variety of genomes, complete chromosomes, contiged sequence maps, and integrated genetic and physical maps.

2.2.5. Molecular Modeling Database (MMDB) (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Structure)

MMDB ***(9)*** contains experimental data from crystallographic and nuclear magnetic resonance (NMR) structure determinations obtained from the PDB. The data in MMDB is crosslinked to bibliographic information, to the sequence databases, and to the NCBI taxonomy. Cn3D is a 3-D-structure viewer for the molecular models, which can be downloaded free of charge.

2.2.6. PopSet Database (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Popset)

The PopSet database contains aligned sequences, submitted as a set, resulting from a population, a phylogenetic, or mutation study describing such events as evolution and population variation. The PopSet database contains both nucleotide and protein sequence data. Currently, nuclear receptors are not represented.

2.3. PIR (http://pir.georgetown.edu)

Protein Information Resource ***(10)***, in collaboration with the Munich Information Center for Protein Sequences (MIPS) ***(11)*** (http://www.mips.biochem.mpg.de) and the Japan International Protein Sequence Database, produces the PIR-International Protein Sequence Database (PSD), the largest, most comprehensive, annotated protein sequence database in the public domain. PIR files can be accessed through the Entrez system at GenBank, but these are local files rather than a direct link to PIR. The search engines at the PIR website are particularly useful for domain and gene family searches. Data is organized into the following databases.

2.3.1. PSD and PATCHX (http://pir.georgetown.edu/pirwww/dbinfo/patchx.html)

PATCHX consists of nonredundant, publicly available protein sequences not yet in the PIR-International PSD. PIR+PATCHX, a combination of

the PSD and PATCHX, contains ~300,000 sequences available for similarity searches.

2.3.2. ARCHIVE (http://pir.georgetown.edu/pirwww/dbinfo/archive.html)

Archive is a database of protein sequences taken directly from published articles or from direct submission, the only such collection of "as published" unmerged sequences.

2.3.3. NRL_3D (http://pir.georgetown.edu/pirwww/dbinfo/nrl3d.html)

This database consists of 3-D structures produced from entries in the PDB.

2.3.4. FAMBASE (http://pir.georgetown.edu/pirwww/dbinfo/fambase.html)

FAMBASE contains representative sequences from each protein family, which can be used in a similarity search to reduce search time and improve sensitivity for identifying distant families. Developed from the PROT-FAM database at MIPS (http://vms.mips.biochem.mpg.de/mips/programs/classification.html) (*see* **Note 3**).

2.3.5. PIR-ALN (http://pir.georgetown.edu/pirwww/dbinfo/piraln.html)

PIR-ALN is a database of curated sequence alignments and consensus patterns ***(12)*** (*see* **Note 3**).

2.3.6. RESID (http://pir.georgetown.edu/pirwww/dbinfo/resid.html)

RESID contains information on posttranslational modifications with descriptive, chemical, structural, and bibliographic information based on features in the PSD.

2.3.7. ProClass (http://pir.georgetown.edu/gfserver/proclass.html)

ProClass consists of nonredundant PIR-International PSD and SWISS-PROT sequences organized according to PIR superfamilies and PROSITE patterns ***(13)***. *See also i*ProClass (http://pir.georgetown.edu/iproclass).

*2.4. Expert Protein Analysis System (ExPASy) (http://www.expasy.ch) (***see *Note 4)***

ExPASy is the proteomics server of the Swiss Institute of Bioinformatics (SIB). The server is dedicated to the analysis of protein sequences and structures, as well as 2-D polyacrylamide gel electrophoresis.

*2.4.1. SWISS-PROT (http://www.expasy.ch) (*see ***Note 4****)*

SWISS-PROT is a highly annotated, curated protein sequence database with a high level of integration with other databases ***(14)***. SWISS-PROT is a partnership between the EMBL and the SIB. TrEMBL is a computer-annotated supplement to SWISS-PROT, and it consists of entries in SWISS-PROT-like format derived from the translation of all coding sequences in the EMBL Nucleotide Sequence Database, except the coding sequences already included in SWISS-PROT. The Human Proteomics Initiative is a major project to annotate all known human protein sequences.

*2.4.2. PROSITE (http://www.expasy.ch) (*see ***Note 4****)*

PROSITE is a database of protein families and domains, including biologically significant sites, patterns, and profiles ***(15)***, and is particularly useful to determine whether a new protein belongs to a known protein family.

*2.4.3. SWISS-3D IMAGE (http://www.expasy.ch) (*see ***Note 4****)*

This is a database of 3-D images of proteins and other biological macromolecules ***(16)***.

2.4.4. SWISS-MODEL Repository (http://www.expasy.ch/swissmod/SM_3DCrunch.html)

The SWISS-MODEL Repository contains automatically generated protein models. The models were generated by a project termed "3Dcrunch," which modeled all entries in SWISS-PROT against all known protein structures (***17***,***18***; *see* **Note 5** and **Subheading 3.2.3.**).

2.5. European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk)

The EBI is an outstation of the EMBL. As well as maintaining a number of databases, the EBI is engaged in an extensive program of applied research and development on integration and interoperation of biological databases.

EBI's Sequence Retrieval System (http://srs.ebi.ac.uk) integrates the main nucleotide and protein databases, and other specialized databases. BLITZ, FASTA, and BLAST are available for sequence-similarity searching. Sequence analysis programs offered include ClustalW for multiple sequence alignment and inference of phylogenies; GeneMark for gene prediction and PRATT for pattern searching and discovery.

2.5.1. EMBL http://www.ebi.ac.uk/embl/index.html

The EMBL Nucleotide Sequence Database is Europe's primary nucleotide sequence resource ***(5)***. In collaboration with DDBJ and GenBank, the database is produced, maintained, and distributed at the EBI.

2.5.2. Macromolecular Structure Database (MSD) http://msd.ebi.ac.uk/

This is the European equivalent of PDB in the United States, and it is dedicated to the management and distribution of data on macromolecular structures. A core part of MSD is a database of probable quaternary structures (PQS) based on data in PDB. PDB files of X-ray crystallography structures usually contain the contents of the unit cell. This may represent only part of the biologically relevant structure, or multiple copies of the structure. MSD provides access to PQS for these macromolecules.

2.6. Sanger Centre (http://www.sanger.ac.uk)

The Sanger Centre provides integrated efforts in the UK for mapping and sequencing the human genome, and genomes of other organisms. It runs a sequence retrieval system that searches local databases; BLAST searches can be run at the site. It is located on the same campus as EBI.

2.7. Center for Information Biology (CIB) (http://www.cib.nig.ac.jp)

The Center for Information Biology runs DDBJ. The center collects primarily Japanese DNA sequences that are shared with EMBL and GenBank. Although resources for English speakers are somewhat limited, the site does have some interesting sequence display and analysis features.

2.7.1. Protein Mutant Database http://pmd.ddbj.nig.ac.jp/~pmd/pmd.html

The Protein Mutant Database (PMD) is a compilation of natural and artificial mutants for all proteins except members of the globin and immunoglobulin families. It can be searched by a text term or by protein sequence ***(19)***. The output of a sequence search is an alignment of protein sequences with the mutant amino acid highlighted.

2.8. Research Collaboratory for Structural Bioinformatics (RCSB) (http://www.rcsb.org/index.html)

RCSB is a consortium consisting of Rutgers University, San Diego Supercomputer Center at the University of California, San Diego, and the National Institutes of Standards and Technology. It is dedicated to the study of the 3-D structures of biological macromolecules. It runs the protein data bank, as well as other less well-known databases.

2.8.1. Protein Data Bank (http://www.rcsb.org/pdb)

The PDB is the single international repository for the processing and distribution of 3-D macromolecular structure data primarily determined by X-ray

crystallography and NMR ***(20)***. The Structure Explorer search tool can be used to search for relevant PDB entries. Both 2-D and 3-D images can be viewed. It has a good set of links to programs and other websites that can be used to visualize structures. Currently, the links only go to the home pages of these sites, rather than to the relevant PDB entry at the site. It hosts various search and analysis programs, such as MOOSE property finder, a structural alignment program, a Microsoft Windows program to interrogate the 3-D structure of biological macromolecules as found in the PDB (WPDB Database for PC), and AutoDock for docking flexible ligands to macromolecules.

2.8.2. Nucleic Acid Database (NDB) (http://ndbserver.rutgers.edu/NDB/ndb.html)

The NDB, a nucleic acids structural database ***(21)***, and the related databases, the DNA-Binding Protein Database and the NMR Nucleic Acids Database, can be searched using Structure Finder (http://ndbserver.rutgers.edu/NDB/structure-finder/index.html).

2.9. TRANSFAC (http://transfac.gbf.de/TRANSFAC)

TRANSFAC catalogs the genomic binding sites and DNA-binding profiles of transcription factors ***(22)***. Of particular interest is PathoDB, a module of TRANSFAC that deals with pathologically relevant mutations in regulatory regions and transcription factor genes. TRANSFAC is being functionally integrated with two other databases, TRANSPATH (signal transduction) and CYTOMER (organs and cell types).

2.10. Object-Oriented Transcription Factors Database (ooTFD) (http://www.ifti.org)

ooTFD is an object-oriented successor to the transcription factors database ***(23)***. The database contains useful information on DNA binding sites and protein–protein interactions of nuclear receptors and transcription factors in general.

2.11. Database of Interacting Proteins (http://dip.doe-mbi.ucla.edu)

Database of Interacting Proteins is a database that documents experimentally determined protein–protein interactions ***(24)***. Although potentially useful, it currently has little information on steroid receptors. The database is linked to by SWISS-PROT.

2.12. ProDom and ProDom-CG (http://www.toulouse.inra.fr/prodom.html)

ProDom is a database of domain families generated automatically from the SWISS-PROT and TrEMBL ***(25)***. ProDom-CG results from a similar domain

analysis of fully sequenced genomes. The families are built using a novel procedure based on recursive PSI-BLAST searches ***(6,26)***. The database has automated URL linking to a number of databases including SWISS-PROT, PDB, Pfam-A and Prosite. The NRR (http://nrr.georgetown.edu/nrr/nrr.html) contains a useful set of links that provide a convenient entry point into ProDom.

2.13. Parallel Protein Information Analysis System (PAPIA) (http://pdap1.trc.rwcp.or.jp/papia/PAPIA.html)

PAPIA is a 2-D and 3-D protein analysis system that uses a parallel array of Intel-driven computers (currently 64 × 200 MHz). One of its main uses is to search the PDB database of structures with either one's own structure file or another file in PDB. The output is a list of related structures that can be visualized using Chime or a Java applet (*see* **Note 6**).

3. Methods

3.1. Analysis of Unknown DNA Sequence

3.1.1. Nucleotide Sequence Search at NCBI Using BLAST

Go to http://www.ncbi.nlm.nih.gov:80/BLAST/ and select "Standard nucleotide-nucleotide BLAST." Enter the following in the search box:

aattacaaagattgcaggtatcctatgaagagtatctctcattgaaaaccttactgcttc

Press the "BLAST!" button. A request ID is returned. Press "Format!" A new window will open, and the results of the search will be displayed when the search is complete.

3.1.2. Analysis of the Results

Results of the search are presented in five sections. First, there is information on the search itself, followed by a graphical representation of the results, then a descriptive table of the matches, a pairwise alignment of the query with matching sequences from the database, and finally, statistics on the search. Mouse-over the sequences represented as purple lines. Note the name of the sequence in the message box. Scroll down and compare the table to the graphical output.

3.1.2.1. Alignments

Click on the purple line representing the rat glucocorticoid receptor (GR). The page scrolls to show the alignment of the query with the rat GR. Note that the sequences are identical, except for three nucleotides. Scroll up and down the page to view other results. Note that the mouse GR also matches the query, except for the same three nucleotides.

3.1.2.2. Query-Anchored with Identities Format

Go to the formatting window, and in the pull-down bar, select "Query-anchored with Identities." Now press "Format!" A new window opens and the results are shown immediately. These results are the same as those viewed previously, but in a different format. Scroll down the page to view the alignment of sequences. In this output, sequences are compared in one large table, rather than in a pairwise view. This view can be particularly informative because it allows all related sequences to be compared at a glance.

3.1.3. Linkage to GenBank

Click on the Y12264 link. This is an entry for the *Rattus norvegicus* glucocorticoid receptor mRNA. Scroll down and view the file. Go back to the top and select "Protein" (in blue). The protein entry for this nucleotide sequence is listed. Select "Related Sequences" (in blue) to give related protein sequences in the database.

3.1.4. Linkage to SWISS-PROT Entry at NCBI

Select the entry, "P06536." This is a SWISS-PROT file maintained at NCBI. SWISS-PROT entries are particularly useful because they are, in general, nonredundant. For instance, this is the only SWISS-PROT file for the rat GR. Note that the file contains links to MedLine (PubMed) and to GenBank entries.

3.1.5. Linkage to the Original SWISS-PROT File

To view the original SWISS-PROT file at the SWISS-PROT database, open a new browser page and go to: http://expasy.cbr.nrc.ca/cgi-bin/get-sprot-entry?P06536. Notice that this entry is better annotated than the one at NCBI. Select the NiceProt link, and examine the page. Once you have finished examining the NiceProt view, close the browser window, and go back to the SWISS-PROT file at NCBI.

3.1.6. Linkage to PubMed

From the SWISS-PROT file P06536 at NCBI, select the link:

JOURNAL	Nature 352 (6335), 497–505 (1991)
MEDLINE	91326070
REMARK	X-RAY CRYSTALLOGRAPHY OF 440–525.

The PubMed literature citation that appears reports the 3-D structure of the GR DNA-binding domain (DBD).

3.1.7. Related Articles in PubMed

Select the "Related Articles" link in blue. A list of related articles, generated by a computer-scoring matrix, opens. The original article appears at the top, followed by other articles in descending order of relatedness.

3.1.8. Linkage to Structure

Select the "Structure" link (in blue, on the right of the page) of the first article. When a page of structural links appears, select "1GLU." The entry for the structure of the GR in the MMDB is shown. Use of the MMDB is discussed in **Subheading 3.2.1.8.**

3.2. Structural Analysis of Nuclear Receptors

3.2.1. 3-D Models

An understanding of the mechanism of action of nuclear receptors requires an understanding of their 3-D structure. The crystal structures of the DBD and the hormone-binding domain of a few receptors have been solved. Given the structural similarity between the receptors so far analyzed, reasonable models can be built for the others. However, such models, at best, give only an approximation of the true structure of the protein being modeled. In this protocol, the authors display and manipulate 3-D structures determined from X-ray crystallography, examine models of nuclear receptors, and finally use the SWISS-MODEL resource to build a model of the GR DBD, then compare it with the experimentally determined structure of this protein.

3.2.1.1. Search PDB for Glucocorticoid Structures

Go to: http://www.rcsb.org/pdb/, which is the home page of the PDB. Under search options, select "SearchLite," then enter "glucocorticoid receptor" and press the search button. A list of NMR and X-ray crystal structures appears.

3.2.1.2. Explore 1GLU

The "Explore" link is on the righthand side of 1GLU, which is an X-ray structure of the GR DBD. This structure was used in **Subheading 3.1.8.**, where the MMDB version of this file was examined. First, examine the structure using a number of tools, starting with Quick PDB, then in **Subheading 3.2.1.8.**, explore the structure as presented in MMDB at NCBI.

3.2.1.3. QuickPDB

Select the option "View Structure," then select the button "QuickPDB." This is a Java applet and only requires that the browser is Java-enabled. The

structure opens in two windows. The top window lists the protein sequence; the bottom window shows the structure. Since this structure is a dimer, there are two protein sequences. Two DNA sequences are shown in the top window, but they cannot be visualized in the 3-D model. The zinc molecules, which are part of the structure, cannot be seen because the 3-D model does not display ligands.

Click on the protein sequence, "CGSCKV," in the top polypeptide; note that the corresponding region in the 3-D model is shown. Rotate the molecule by clicking and moving your mouse. Note that the CGSCKV sequence that determines DNA-binding specificity (knuckle, P-Box) is at the end of an α-helix. Once you are familiar with the limited capabilities of QuickPDB, close the applet window and select FirstGlance in the browser window.

3.2.1.4. FirstGlance

FirstGlance requires installation of the Chime plug-in, which only works with Netscape Navigator with the MacOS (*see* **Note 7**). Once Chime is installed, select the "FirstGlance" option. 1GLU opens as a rotating dimer bound to DNA. Use the checkboxes to change the view. On the bottom right of the page are the letters "MDL." Click on these to obtain a menu of options. The use of Chime is beyond the scope of this chapter. The tutorials and help files are particularly informative and can be found at http://www.mdli.com/support/chime/demo2.html.

3.2.1.5. Protein Explorer (http://www.rcsb.org/pdb/pe/explorer/pe.htm?id=1GLU)

Protein Explorer is another Chime implementation. It has more features than FirstGlance, in that it has a command-line interface, and multiple molecules can be loaded into the program. Once the image has been manipulated, go back to the 1GLU PDB structure page and select Protein Explorer again. However, this time, when asked for a second molecule, enter 1A6Y (ReverbA) ***(27)***. Note that to manipulate the images, one must select either the "Top" or "Bottom" radio button first (in red at the top). Manipulate both images. The GR can be displayed so that it appears in one plane to the DNA and is present as a homodimer in a head-to-head configuration. The ReverbA structure, however, is a tandem homodimer that curves around the DNA.

3.2.1.6. Virtual Reality Modeling Language

There are two options for virtual reality viewing. One uses default options, an interactive immersive ribbon diagram, the other uses Virtual Reality Modeling Language (VRML), which gives a full-screen display and allows customization of viewing parameters. Both options require a VRML plug-in for the browser (*see* **Note 8**).

Once a virtual reality plug-in has been installed, select the default VRML view (*see* **Note 9**). If using Cosmo Player, click on the small "?" on the bottom right to get instructions on how to use the viewer. The model can be spun by clicking and moving the mouse in the direction of desired spin. The recognition helices are easily discerned, as is the dimer interface. The view is limited, however, since one can only move around the image, details of the view itself are not modifiable.

Switch to the customizable VRML view, and view with the default settings (*see* **Note 10**). Find the recognition helices. Go back to the settings control and select "Draw bases." View the model, then go back to the settings control and select "Draw all sidechains." Although the picture is now visually arresting, it lacks hydrogen bonds and a method to measure distances between atoms.

3.2.1.7. STING/GRASS/GRASP (HTTP://TRANTOR.BIOC.COLUMBIA.EDU/)

In the PDB 1GLU file, select "Other Sources." A whole host of links is shown. For 3-D modeling, the author particularly likes the presentation of structure by STING and GRASS.

STING is a PDB 3-D structure browser (http://trantor.bioc.columbia.edu/STING; for 1GLU, http://trantor.bioc.columbia.edu/cgi-bin/STING/frame.pl?1glu). A useful program also available at this site is STING paint, a program that colors protein sequence alignments (http://trantor.bioc.columbia.edu/STING/STINGpaint).

GRASS is an online implementation of GRASP (Graphical Representation and Analysis of Structural Properties) (http://trantor.bioc.columbia.edu/GRASS/surfserv_frms.cgi?1glu) (*see* **Note 11**). Static images generated by Grasp for 1GLU can be found at:

http://trantor.bioc.columbia.edu/GRASS/surfserv_picgallery.cgi?1glu

3.2.1.8. MMDB (HTTP://WWW.NCBI.NLM.NIH.GOV:80/ENTREZ/QUERY.FCGI?DB=STRUCTURE)

The molecular-modeling database at the NCBI is particularly useful because it is integrated into the Entrez system. In the example used in **Subheading 3.1.**, the author eventually arrived at the MMDB entry for the rat GR DBD, derived from the PDB structure 1GLU. MMDB can also be reached using the link on the PDB "Other Sources" page of the 1GLU entry: MMDB uses its own 3-D viewer, Cn3D. Download it from the links provided (*see* **Note 12**).

3.2.1.9. VIEWING 1GLU IN CN3D

Go to: http://www.ncbi.nlm.nih.gov/cgi-bin/Structure/mmdbsrv?form=6&db=t&Dopt=s&uid=1glu. Once Cn3D has been set up, press the view button using the default parameters. Cn3D should open with two windows: an upper

window showing the 3-D structure; and a lower window showing the protein sequence with annotations of structural elements. The two windows are interconnected: Colors of the sequence in one window are identical to those in the other window. This feature allows easy correlation of residues in the sequence with those in the structure. Dragging and clicking the mouse across a region of the sequence will highlight the letters, and will apply the same highlight color to the corresponding amino acids in the 3-D window. Manipulate the sequence (if necessary, use the reset command under the view menu). Select the "CGSCKV" sequence and note how it lights up in the 3-D model.

3.2.1.10. Viewing Structural Neighbors Using Vector Alignment Search Tool (VAST) ***(28)***

http://www.ncbi.nlm.nih.gov/Structure/VAST/vast.shtml

The VAST algorithm determines protein structure neighbors by direct comparison of 3-D protein structures in MMDB. In this analysis, each one of the domains in MMDB is compared to every other domain (more than 18,000 domains in all). Cn3D can be used to display these aligned protein sequences, both as linear sequence and as 3-D models.

To view a structural neighbor of the GR DBD, use the VAST. The MMDB entry for 1GLU, discussed above, has links for structural neighbors. Select the link marked "B." Check the box for "Retinoid X Receptor-Thyroid Hormone Receptor DNA-Binding Domain Heterodimer Bound To Thyroid Response Element DNA" and press the view button using the defaults.

Cn3D should open and the two 3-D structures should be aligned. By default, red indicates alignment, and blue, unaligned regions. The sequence window shows the sequence alignment. Highlight the sequence, CGSCKV; the corresponding sequence in the recognition helix of the model becomes highlighted. Note how the peptide backbone of the α-helix is well maintained, i.e., DNA-binding specificity is the result of the amino acid side chains, rather than a change in the overall structure of the protein. Explore the display options of Cn3D.

3.2.2. Homology Modeling

In homology modeling, the 3-D structure of a protein is predicted based on its similarity to a protein of experimentally determined structure. This technique assumes that the backbone of the protein and the similar one of known structure (i.e., the template) are identical. Thus, the sequence similarity between the two proteins must be significant. If there is no similar structure in PDB, the 3-D structure of an unknown protein cannot be predicted by any current protocol.

3.2.2.1. SWISS-MODEL ***(17,18)*** (HTTP://WWW.EXPASY.CH/SWISSMOD)

SWISS-MODEL is an automated protein-modeling server running at the Glaxo Wellcome Experimental Research Center in Geneva, Switzerland. The self-described purpose of the server is to make protein modeling accessible to all biochemists and molecular biologists worldwide. To model a protein, one or more ExPDB templates are used. ExPDB files are derived from PDB files: Each chain is in a separate file, and the residues have been renumbered continuously. The models themselves are constructed using ProModII and Gromos96 (energy-minimization) programs.

3.2.2.2. MODELING THE DNA BINDING DOMAIN OF GR BASED ON THE THYROID HORMONE RECEPTOR DNA BINDING DOMAIN STRUCTURE

The 3-D structures of the rat GR and the thyroid hormone receptor (TR) DBDs have been solved using X-ray crystallography. In this example, the authors create a theoretical model of the GR DBD using the TR DBD template, then compare the theoretical GR structure with the crystal structure.

Go to the SWISS-MODEL home page: http://www.expasy.ch/swissmod/.

Select the "First Approach Method" and fill out the form with specific information, and use P06536 for the SWISS-PROT accession number (rGR whole protein sequence), and 2NLLB for the ExPDB template (TR DBD).

Submit the sequence using the defaults. While waiting for the results to be sent by e-mail, set up SWISS-PDB viewer.

3.2.2.3. SWISS-PDB VIEWER ***(18)*** (HTTP://WWW.EXPASY.CH/SPDBV/MAINPAGE.HTM)

Download and install (http://www.expasy.ch/spdbv/). Set up the e-mail program to open attachments of the type "chemical/pdb" with SWISS-PDB viewer (*see* **Note 13**). If you are unfamiliar with SWISS-PDB viewer, the tutorial is well worth the time, although for this demonstration, it should not be needed. Open the file with SWISS-PDB viewer. Close any log files. Arrange screen so that three windows can be seen: a central structure viewer, a control panel, and a tool bar (if the tool bar is not open, open it from the window menu [Wind]). You may also wish to open the 2-D viewer window (Sequences alignment) from the window menu (Wind).

3.2.2.4. ANALYSIS OF THE MODEL USING SWISS-PDB VIEWER

1. Turn off the display of all side chains and the ribbon (shift-click a check mark under "side" and under "ribn").
2. Set the color of all amino acids to red (shift-click a check box under "col").
3. Under menu "Display," select "Show CA trace only."

4. Select 2NLLB in the control panel (click on Target, and select 2NLLB) and deselect "Visible."

The GR DBD should now appear as a single red line with the α-helices visible on rotation (use the toolbar to set controls). The structure on view is a theoretical model of the GR based on the TR DBD. Next, the authentic GR X-ray crystal structure needs to be loaded so that the theoretical and X-ray structure can be compared.

3.2.2.5. Comparison of GR Model with X-ray Structure

1. Import the 1GLU PDB file using the import function under the File menu (*see* **Note 14**).
2. Press the "=" key to center and view molecules.

1GLU contains two GR chains and a DNA molecule so the next job is to simplify the view.

1. Make 1GLU active in the control panel (select 1GLU at the top of the panel).
2. Turn off side chains, and if necessary, the ribbon (shift-click a check mark under "side" and under "ribn").
3. Set all residues to blue (shift-click a check box under "col").

There are four molecules in the structure: A, B, C, D (A and B are protein, C and D are DNA).

1. Turn off display of the B, C, and D chains (scroll down in the control box and deselect the "show" check-mark for the B, C, and D chains).
2. Color Cys457 green, Phe464 yellow, Cys476 purple.
3. Switch the control panel to the "Target" sequence.
4. Color Cys457 green, Phe464 yellow, Cys476 purple.
5. Arrange the molecules so that the colored amino acids are clearly visible. If the molecules appear to be different sizes, rotate them so that they are in the same plane; press "=" to center the view.
6. On the tool bar, select the merge function (the button with red and green dots and an arrow between them). Follow the instructions on the tool bar in the following order:
   a. Green amino acid on blue chain
   b. Green amino acid on red chain
   c. Yellow amino acid on blue chain
   d. Yellow amino acid on red chain
   e. Purple amino acid on blue chain
   f. Purple amino acid on red chain

The view can now be rotated; side chains, DNA, and other chains can be made visible if one wishes. The purpose of this exercise is to gain an idea of how well the modeling system works. As can be seen from the structures, there is good overall alignment of the structures, but there is also significant deviation (*see* **Subheading 3.2.3.3.**).

3.2.3. Using SWISS-MODEL Repository (3Dcrunch Database) (http://www.expasy.ch/swissmod/SM_3DCrunch.html)

The SWISS-MODEL Repository contains automatically generated protein models from the 3Dcrunch project, which modeled all entries in SWISS-PROT against all known protein structures in PDB (*see* **Note 5**). In this example, the authors use this database to view models of the GR DBD.

3.2.3.1. Searching the Repository

Go to: http://www.expasy.ch/swissmod/SM_3DCrunch_Search.html. Enter the SWISS-PROT code P06536 (rat GR, complete protein) and search. In the reply, select the entry C00002, residue range 553–633 (this is a model of the DBD of the rat GR) and submit. The results are sent back by e-mail.

3.2.3.2. Displaying Results in SWISS-PDB Viewer

Open the e-mail attachment in SWISS-PDB viewer (*see* **Note 13**). If this program has not been used before, *see* **Subheading 3.2.2.1.** The alignment of six models is shown.

1. Close the log window.
2. Open the 2-D alignment window (sequences alignment) from the window (Wind) menu.
3. Using the control panel, deselect the ribbon option and hide side chains as detailed in **Subheading 3.2.2.5.**

1GLUB and 1GLUA are individual chains from the X-ray crystallographic structure 1GLU of the GR DBD; 1GDC, 2GDA, and 1RGD are NMR-derived structures of the GR DBD.

3.2.3.3. Comparing GR DBD Model with Crystal Structure

Using the control panel, make all chains invisible except "Target" and 1GLUB. Select the 3-D model window and press "=" to center the image. Rotate the image to view the alignment. To get an idea of how far apart divergent atoms are, select the measurement tool, and click on equivalent atoms. The two models place most atoms to within 1 Å, but some are over 4 Å apart. Guez et al. ***(17)*** compared 1200 model–control structure pairs and calculated the relative mean square deviation (rmsd [based on Cα atoms]) of the models from their controls. Below 30% sequence identity, only 10% had a rmsd of ≤2Å. Above 80% sequence identity, ~79% had a rmsd of ≤2Å and over 40% had a rmsd of ≤1Å. These results show that great care should be exercised when using protein models, especially when the protein sequence identity is low.

3.3. Obtaining Background Information on Nuclear Receptors

3.3.1. Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm.nih.gov/Omim

OMIM is a comprehensive and constantly updated database of inherited diseases *(29)*. If a gene of interest is in OMIM, this is the place to start a literature search. Each entry is a minireview on the gene and its pathology. The articles in OMIM are particularly well linked, so that a large amount of information can be obtained easily and quickly. Within OMIM, articles are cross-referenced, and OMIM has its own gene map, which allows scanning the chromosome near a gene of interest. Linkage to the Entrez system includes individual PubMed articles, all papers in an article presented in a PubMed window, a link that brings up DNA sequence entries from GenBank, and another that brings up protein sequences. The Genome database entries are linked, as are LocusLink *(30)* entries. Outside links include the Genome Database, the Mouse Genome Database, the Human Nomenclature Database, and the Coriell Cell Repositories of cell lines from patients with genetic disease.

3.3.2. Nuclear Receptor Resource http://nrr.georgetown.edu/nrr/nrr.html

As discussed in **Subheading 2.1.1.**, the NRR is a database that focuses on nuclear receptors. It has a wealth of information on these proteins, links to other databases, and has a list of scientists that work on nuclear receptors, and jobs available/wanted, and so on.

4. Notes

1. The focus of this article is the analysis of nuclear receptors; thus, only those databases that were deemed essential for this purpose are discussed. Because of space limitations, many WWW sites that have useful information have been omitted A list of databases can be found at: http://nar.oupjournals.org/cgi/content/full/28/1/1/DC1.
2. The NRR consists of the Glucocorticoid Receptor Resource, the Thyroid Hormone Receptor Resource, Androgen Receptor Resource, the Androgen Receptor Mutation Database, the Mineralocorticoid Receptor Resource, the Vitamin D Receptor Resource, the Peroxisome Proliferator Activated Receptor Resource, and the Steroid Receptor Associated Proteins Resource. All components of the NRR can be accessed from the NRR home page: http://nrr.georgetown.edu/nrr/nrr.html.
3. Alignment of protein sequences, and use of the alignments to generate protein families, differs from database to database. A description of the system used at PIR *(12)* can be found at: http://pir.georgetown.edu/pirwww/aboutpir/doc/short_sf_def.html.

4. The ExPASy server has a number of mirrors worldwide. Because the links in the home page currently rotate through the mirror sites, only the home page URLs are shown here:
   a. Switzerland: http://www.expasy.ch/ at Swiss Institute of Bioinformatics, Geneva.
   b. Australia: http://expasy.proteome.org.au/ at Australian Proteome Analysis Facility, Sydney.
   c. Canada: http://expasy.cbr.nrc.ca/ at Canadian Bioinformatics Resource, Halifax.
   d. China: http://expasy.pku.edu.cn/ at Peking University.
   e. Taiwan: http://expasy.nhri.org.tw/ at National Health Research Institutes, Taipei.
5. The 3Dcrunch project was run in 1998, and so contains models based on structures and sequences in the PDB and SWISS-PROT databases at that time. New sequences and structures are added to the database periodically ***(17)***, however, explicit update information is not given on the database's home page.
6. The lists of files that are produced by PAPIA have links to an Applet viewer. Unfortunately, the viewer crashes on both Netscape and Internet explorer browsers for the Mac OS. The output also has a link for a RasMol (RasMac) output; this works fine with Netscape but fails with Internet Explorer for the Mac.
7. Chime home page: http://www.mdli.com/support/chime/default.html.
8. The author has good luck with Cosmo Player for Netscape Navigator with the Mac OS. The ExpressVR/Internet Explorer combination works less well. A list of Mac OS plug-ins can be found at http://www.macorchard.com/vrml.html.
9. The following link leads directly to the VRML presentation: http://www.rcsb.org/pdb/cgi/explore.cgi?job=graphics&pdbId=1GLU&page=0&pid=7771965152037&opt=vrml_default.
10. The following link leads directly to the VRML presentation: http://www.rcsb.org/pdb/cgi/explore.cgi?job=graphics&pdbId=1GLU&page=0&pid=7771965152037&opt=vrml_custom.
11. *See also* http://honiglab.cpmc.columbia.edu/grasp/.
12. http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.html.
13. It is sometimes difficult to set up mail programs to open attachments automatically or to even save them as the correct file type. On the Mac, if the attachment will not open, save it to disk and use a "file type changer" utility to change the type of file to TEXT and the creator to "P3D√." Programs that can do this include:
   a. Norton Disk Editor (open the disk, select the file, then select "Get Info").
   b. FileTyper (Mac OS): http://www.ugcs.caltech.edu/%7Edazuma/filetyper/index.html.
   c. DLTypes v3.0 (Windows), available from http://www.zdnet.com/downloads/powertools/, does a similar job on the PC.
14. To import via the WWW, set the server to 213.39.71.210 port 27000 in the network preference panel under the "Prefs" menu. If this fails, visit http://www.usm.maine.edu/spdbv/text/server.htm.

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2

Phylogenetic Inference and Parsimony Analysis

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1. Introduction

Application of phylogenetic inference methods to comparative endocrinology studies has provided researchers with a new set of tools to aid in understanding the evolution and distribution of gene families. Phylogeny, as defined by Hillis et al. ***(1)***, is the "historical relationships among lineages of organisms or their parts (e.g., genes)." Inferring phylogeny is a way of generating a best estimate of the evolutionary history of organisms (or gene families), based on the information (often incomplete, as in a gene sequence) that is available. The use of phylogenetic analyses, specifically those methods that are based on maximum parsimony, has changed the way in which characters and character states are determined and interpreted. Maximum parsimony (often simply called "parsimony") seeks to estimate a parameter based on the minimum number of events required to explain the data. In this type of phylogenetic analysis, the best or optimal tree (generally portrayed as either a cladogram or phylogram, *see* **Note 1**) is that topology which requires the fewest number of character-state changes (*see* below). That tree is arrived at based upon consideration of shared, derived characters. This method assumes that when two taxa (or genes) share a homologous derived character state, they do so because a common ancestor of both had that character state. One goal of phylogenetic analysis that is always implied (and often stated) is to avoid using characters that are homoplastic. Characters that have homoplasy have similarities in character states for reasons other than inheritance from a common ancestor, including convergent and parallel evolution or a reversal of state (e.g., A → G → A).

The most common types of molecular characters that are used in phylogenetic analysis of steroid hormone receptors are the primary sequence positions of DNA or proteins, cDNA sequences derived from RNA, and amino acid

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sequences of proteins inferred from cDNAs. Therefore, in most situations phylogenetic analysis of these sequences is virtually identical to the analysis of sequences in a molecular systematics study attempting to resolve relationships among different taxa. In this chapter, a number of the most commonly applied methods of analyzing such data sets are introduced, emphasizing the phylogenetic approach using parsimony. Although parsimony-based models are emphasized here, other approaches such as maximum likelihood, can also be used for nucleotide-based ***(2)*** or amino acid based ***(3,4)*** phylogeny reconstruction. Maximum likelihood methods are used to evaluate a hypothesis about evolutionary history based on the probability that the proposed model of the evolutionary process and hypothesized history would give rise to the observed data ***(5)***. There are also a number of phenetic approaches (those based on overall character similarity, e.g., unweighted pair group method with averages), some of which are sometimes considered to be more or less phylogenetic methods (e.g., neighbor joining) ***(6)***. All phenetically-based trees (called phenograms) are ultimately generated from similarity measures that are used to estimate genetic distances. Application of these methods certainly may have merit for some studies of steroid hormone receptors, and although the criteria for recovering the sequences and their alignment are literally the same for all of these methods, this discussion is restricted to phylogenetic analyses that are based on maximum parsimony.

Phylogenetic analysis deals with both characters and character states. As noted above, molecular characters are usually the positions of the nucleotides of the DNAs or amino acids for the proteins that are being compared. Virtually all sequence analyses lead to the generation of multistate characters; for nucleotide-based data sets, the character states are normally A, G, C, or T (although a fifth state, which accounts for missing bases, is also often included); for protein data sets, the states would then be the 20 naturally occurring amino acids (again, a state for a gap character could also be included). Multistate characters may be ordered or unordered: They are said to be ordered if a particular state exists between two states (e.g., if mutation to T were required as an intermediate condition during a change from G to A). This requirement is virtually never observed in molecular data, so it is assumed that most nucleotide or amino acid sequence data sets are both multistate and unordered (indicating any state can be reached from any other state).

Homology (inferred common ancestry of genes or gene products) is the characteristic that actually allows one to compare sequences. The two most important types of homology in most molecular data should be distinguished. Orthology assumes that the common ancestry of two sequences can be traced

back to a speciation event. Paralogy indicates that the common ancestry of the sequences can be traced back to a gene-duplication event.

A series of sequences that are either orthologs (comparing taxa) or paralogs (comparing lineages of genes), and which all share the same common ancestor, are said to be monophyletic. Monophyletic groups can include gene sequences from different members of a genus or species or related sequences of a gene family (e.g., the estrogen β receptors). In any phylogenetic analysis, it is advisable to employ outgroup comparison. The so-called "ingroup" includes members of a taxon (or genes in a lineage), assumed to be monophyletic. The ingroup sequences can be distinguished from sequences outside of it by having a larger number of shared, derived characters (synapomorphies). Related genes (such as estrogen α receptors when compared to estrogen β receptors) or taxa (such as alligators when compared to crocodiles), might have an evolutionary history similar to the ingroup. They would share fewer synapomorphies with the ingroup members, but would share some number of primitive characters (symplesiomorphies) with the ingroup. Inclusion of these outgroup sequences allows for rooting (*see* **Note 2**) of the phylogenetic tree and verification that all members of the ingroup lineage are more closely related to one another than to some other sequence. At least one outgroup sequence should always be employed in phylogenetic analysis, and in some cases it is important to have two or more (*see* below).

At first glance, the use of primary sequence positions as characters for phylogenetic inference might be considered reasonably straightforward. Examining two purportedly homologous sequences, counting the number of bases or amino acids from one terminus and comparing the two sequences (at say amino acid positions 1–65 for some protein), would allow the absolute number of differences between two sequences to be readily ascertained. However, this simplicity may be misleading. In assessing phylogeny, establishing positional homology is critical and can be complicated. In comparing amino acid sequences, having positional homology indicates not only that both sequences are homologous (e.g., both are estrogen β receptors), but also that every amino acid occurring at a particular position in the protein sequences (e.g., amino acid 43) being compared trace their ancestry to a single position that occurred in the protein sequence of a common ancestor ***(5)***. In all but closely related protein genes and/or the most highly conserved sequences, insertions or deletions probably will have occurred in the nucleotide sequences and thus, often in the amino acid sequences. These must be accounted for by alignment to ensure positional homology. Therefore, proper alignment of sequences, considered by many to be the most critical aspect of molecular phylogeny, will be the first method that is addressed (*see* **Subheading 2.**).

2. Materials

Virtually all researchers have their favorite phylogenetic analysis package(s). For all-around versatility with molecular sequence data, Phylogenetic Analysis Using Parsimony (PAUP*) ***(19)***, a package developed by Swofford, is difficult to beat, especially if one has had a MacIntosh computer. Recently, PC-compatible and UNIX versions have joined the VAX/VMS and Mac OS packages. Reasonable ($85–200 for virtually all operating systems) to acquire through Sinauer Associates (orders@sinauer.com) and menu-driven, it is the most popular phylogenetic analysis package for molecular data. It is the package that my lab uses almost exclusively for phylogenetic analyses. PAUP* has a large number of programs besides those that are parsimony-based and will read a wide range of data input files, including Nexus, PHYLIP, and FASTA.

Perhaps even more versatile, but probably not as easy to use is Felsenstein's Phylogenetic Inference Package (PHYLIP) ***(2)***, a broad package of programs that like PAUP* can perform not only parsimony, but also maximum likelihood and distance analyses. The price of PHYLIP is even more attractive than PAUP*, since it can be acquired at no charge by anonymous ftp from: evolution.genetics.washington.edu (in directory pub/phylip), or by accessing the World Wide Web site: (http://evolution.genetics.washington.edu/phylip.html).

An additional service that Felsenstein has provided at the PHYLIP website is a documented list including 175 programs used for reconstructing relationships. These range from more specialized packages that will primarily perform only alignments (e.g., ClustalW, MacVector, and MALIGN), and deal mainly with genetic distance analyses (e.g., MEGA 2B) or maximum likelihood analyses (e.g., MOLPHY or PAML), to those that allow trees to be interactively manipulated (e.g., MacClade). It also lists those packages that contain a large number of applications (such as PAUP*, PHYLIP, Hennig86, VOSTORG). Included in the documentation for each listing are how to acquire the various programs or packages, a general assessment of the analyses each are able to perform, and any cost that will be incurred.

3. Methods

3.1. Alignment

Possibly the most difficult and poorly understood aspect of phylogenetic analysis is alignment. Local alignment algorithms find all matches in a database search above a certain defined threshold (e.g., 50%). Data bank searches, such as those employed by the National Center for Biotechnology Improvement (NCBI) data bank (http://www.ncbi.nlm.nih.gov/), use several of these algorithms. Two examples are BLAST ***(7)*** and FASTA ***(8)***. The program

"Entrez" available at the NCBI address above allows rapid evaluation of both nucleotide and protein databases. Once genes of interest are identified, Entrez allows location of many similar sequences (however, not necessarily homologous). These can be identified by taxonomic group, terms in titles or abstracts of papers, authors, key words, accession numbers from the database, gene names, and so on. Then the best matches can be extracted and aligned prior to phylogenetic analysis.

Pairwise sequence alignment (which seeks to align two entire homologous regions) is accomplished by the inclusion of gaps, which correspond to insertions or deletions, and balancing these with matches. Most sequence alignment programs are ultimately a derivation of the global alignment program originally developed by Needleman and Wunsch ***(9)***. Aligning sequences can be simple or tedious, depending on the levels of sequence divergence. However, it should be recognized that if one uses enough gaps, ultimately any two sequences can be aligned, therefore gap penalties must be assigned. The gap penalties are typically a combination of both the gap number and the size of the gaps. The former are usually penalized more heavily than gap size because there is no reason to assume that insertion/deletion events will necessarily involve short sequences. In protein-coding sequences, gaps leading to frameshifts are more heavily penalized than those leading to single amino acid substitutions. Gap penalties can be assigned for unequal length sequences, although 5' or 3' gap penalties are typically lower than those found internally.

Changes leading to substitutions also confer alignment cost. This cost can be assigned as one value for all changes or can be based on a matrix of different values, the difference in the cost depends on whether the change leads to a transition or transversion (for nucleotides) or how frequent the change is. For protein sequences, different kinds of changes at the amino acid level (e.g., aliphatic to aromatic amino acid, helix former to helix breaker, and so on) can be assigned different alignment costs. Ultimately, regardless of the sequence alignment that is produced by any computer program, the final alignment should only be accepted after visual inspection, which can lead to alignment changes based on secondary levels of structure at either the nucleotide or amino acid level.

In almost every phylogenetic study, more than two sequences are being examined and there is the requirement for multiple sequence alignment. One approach is to make a series of pairwise alignments, then add all the sequences together. The overall alignment is then the sum of each additional step and compensates by inserting gaps as necessary; one caveat is that this approach is dependent on the order in which the sequences are added. Several ways of overcoming the problem of order dependence have been proposed. One method

is to obtain the order of pairwise alignments from clusters in an initial tree generated for a distance matrix across all pairwise alignments ***(10)***. The program called "Clustal" ***(11)*** uses this format, as do several other programs. A similar, but somewhat modified approach is used in the program "TreeAlign" ***(12)***. PILEUP, a program in the Wisconsin Genetics Package sold by the Genetics Computer Group, uses "progressive pairwise alignment" to produce multiple alignments. All are effective, as long as visual inspection verifies the computer-generated alignment.

An alternate strategy is based on the premise that alignment is a constituent part of phylogenetic inference, rather than a treatment that is applied prior to it. The program called "MALIGN" ***(13)*** optimizes multiple alignments by searching for the alignment that minimizes the differences between the sequences. These differences are specified by the defined gap penalties and assigned costs resulting from the substitutions mentioned above. For many studies, the ability of the user to set parameters such as gap weighting and sequence order make this is a very versatile approach. Furthermore, this program outputs aligned sequences that can be used with most all of the major phylogenetic analysis programs.

3.2. Phylogenetic Analysis of Aligned Sequences Using Parsimony

Because most of this discussion is limited to parsimony analysis, it is imperative to identify the important distinctions among the different major types of parsimony and to establish criteria for the use of each, then elaborate on the most widely applied analyses. As stated earlier, parsimony is an optimality approach that seeks to find the minimal tree length. Although there are a number of ways to achieve that goal from the perspective of different algorithms, as Swofford et al. ***(5)*** state, "Algorithms tend to have short life spans," thus, one needs to be driven by the conceptual framework and not by any specific algorithm.

3.2.1. Common Types of Parsimony and Application for Nucleotide Sequences

1. Fitch parsimony is the simplest type of analysis, which imposes no constraints on character state changes. It allows unordered, multistate changes from any one state to any other state with reversibility ***(14)***.
2. Camin–Sokal parsimony allows multistate, unordered changes, but does not allow reversibility ***(15)***.
3. Transversion parsimony. Because of the higher likelihood of transitions (T → C, C → T, A → G, G → A) over transversions (A or G → C or T [and vice versa]), transitions are ignored and only transversions are used as shared, derived charac-

ters (*see* **Note 3**). These can be recoded as either purines or pyrimidines and Wagner parsimony (*see* **Note 4**) applied.

4. Threshold parsimony, a method developed by Felsenstein ***(2)***, prevents rapidly evolving characters from adding enough length to a tree under consideration to cause it to be rejected. This is accomplished by counting the steps each character must have for a given tree, but not applying these above a specified threshold value. For example, if a character state tree requires seven changes, and the imposed threshold is four, then this character only adds four steps to the tree under consideration. Intuitively, this is an attractive method of extracting phylogenetic information in the presence of several rapidly evolving and potentially homoplastic characters.
5. Generalized parsimony, as the name implies, is the most general type of parsimony analysis, but at the same time is computationally expensive (and therefore often slow). This method assigns a cost for each transformation of every character state to all other states. These are set up in the form of a matrix of weights. In concept, it can include transversions in nucleotide sequences, as well as consider amino acid changes that result from several changes at the nucleotide level ***(5)***.

3.2.2. Common Types of Parsimony Application to Protein Sequences

1. Eck–Dayhoff (Fitch) parsimony, as above, is the simplest type of analysis. Here the genetic code is ignored and there is equal probability for any one amino acid to change to any other ***(16)***.
2. Moore–Goodman–Czelusniak (MGC) parsimony seeks trees requiring the fewest number of nucleotide substitutions at the mRNA level ***(17)***. It generalizes the Fitch parsimony approach to codons, incorporating degeneracy of genetic code and guarantees a minimum number of nucleotide substitutions required by any tree (*see* **Note 5**).
3. PROTOPARS is a program developed by Felsenstein ***(2)***, which includes aspects of both Eck and Dayhoff ***(16)*** and Moore–Goodman–Czelusniak ***(17)*** methods. It does not consider silent mutations, although the genetic code is not ignored (*see* **Note 6**).

For studies of nucleotide-based sequences, generalized parsimony and various modifications of transversion parsimony are probably the most widely applied methods. Threshold parsimony is not used as widely (primarily because of a lack of empirical data on threshold values), although it has the potential to be a valuable tool, especially for closely related sequences or those with mutational hotspots. For studies of protein-based sequences, probably the most widely applied parsimony program is PROTOPARS.

3.3. Finding Optimal Trees

When optimality criteria are outlined as in the previous subheading on types of parsimony, essentially a particular tree is being evaluated under a set of

selected criteria (e.g., under transversion parsimony criteria). Finding the optimal tree (or trees) is a different problem, with several approaches that are used to solve it. The most conservative approaches use exact algorithms that typically involve either exhaustive searches or branch and bound searches.

3.3.1. Exact Methods

Exhaustive searches literally evaluate every possible tree topology. In this type of analysis, one starts off with the simplest unrooted association of taxa (three), then adds one taxon per round in all possible combinations (for four taxa, there are three possible trees; for five taxa, 15 possible trees; and so on). This number increases so rapidly that for most studies exhaustive searches are really only practical for eleven or fewer taxa (eleven taxa generate over 35 million possible trees). An advantage of this method is that with all possible trees having been considered, one can look at the frequency distribution of tree lengths (the number of steps required to produce a topology). Near-optimal trees can be identified, so that one can determine whether there are few or many solutions that are close to the most optimal tree ***(5)***.

In most studies, however, even when using a conservative approach to resolve the best tree for the data, it is not necessary to evaluate every single possible topology to find the optimal tree. The so-called "branch-and-bound method" was first applied to phylogenetic analysis by Hendy and Penny ***(18)***. This method adds new groups in all possible combinations, as long as the number of steps involved in the generation of a particular tree is equal to or less than some minimum upper bound of optimality that has been previously chosen. In this way, as new groups are added along a particular branch, if the optimal tree score is exceeded, then the entire branch (from the node that is being evaluated to all terminal groups [located at the ends of branches]) is considered suboptimal (and adding new groups cannot possibly improve the tree score). Thus, no further subsequent consideration along that branch is given (in favor of other branching sequences that do comply with the optimality criterion). In this way, the branch-and-bound still conducts an exhaustive search, but in reality only uses those topologies that can potentially lead to optimal tree resolution. For many data sets of 20 or more gene or amino acid sequences (or taxa), this approach can lead to an exact solution, i.e., a single best tree (or group of trees with identical scores) will be found for that data set.

3.3.2. General Heuristic Methods

Sometimes a data set is so large that the application of exact methods (i.e., exhaustive or branch-and-bound searches) is not practical or feasible in terms of available computing power or time. Then heuristic approaches (*see* **Note 7**) which employ approximate methods can be used. Heuristic tree searches typi-

cally use hill-climbing methods ***(5)***. One tree (randomly chosen) starts the process, then that tree is rearranged in a way that the score is improved to the minimum length. Generally for heuristic searches, one chooses some number replicates (e.g., 100, which will probabilistically evaluate many different starting trees), keeping only the shortest tree(s) found. Often, if the data set has enough information content (i.e., is not too noisy), one will find the optimal tree (or some set of equally optimal trees) that might be recovered in much longer branch-and-bound analyses. There are several ways to accomplish heuristic searches. The most commonly applied algorithms are discussed below.

1. Stepwise addition is a common way of producing a starting point for further rearrangement of taxa (or different sequences) to a growing tree. A simple description of stepwise addition follows. Starting with three taxa for the initial tree, the next taxon is added and each of the three trees that are produced is evaluated and the one with the best score is retained. In the next round, another taxon is added to the tree that was retained from the previous round and the best of these five possible trees is retained for the next round, and so on until all of the terminal taxa are added. A problem with this kind of approach is that while the position of taxon A may be optimal at a particular level of addition, if other taxa are subsequently added later on, it could make taxon A's position suboptimal. Furthermore, if two equally optimal trees exist at a particular level, one really should save both and evaluate each under the stepwise criteria. Not all packages will do this. However, stepwise addition algorithms are rapid and if the data are clean (i.e., little homoplasy), then they will quickly come up with the optimal tree with reasonably high frequency.
2. Branch swapping is a process in which stepwise addition can often be improved by choosing sets of predefined rearrangements. The underlying premise is that if one rearranges the tree(s) that are kept at each round (as in the stepwise addition method), then one of these rearrangements may well lead to a better tree that is more likely to be optimal. The three most commonly employed branch-swapping algorithms are nearest neighbor interchange, subtree pruning and regrafting and tree bisection and reconnection. Each uses a slightly different approach to producing the rearrangement. The scope of the present paper precludes the details of each of these rearrangement types to be presented herein, but with analysis packages like PAUP* ***(19)***, they can be easily accessed in a menu-driven fashion.

3.4. Problems of Systematic and Random Error

Evaluating the error component to any analysis is always critical. In phylogenetic inference, the errors in the analysis are primarily due to either systematic error or random error. Swofford et al. ***(5)*** define random error as the deviation between a parameter of a population and an estimate of that parameter due strictly to the sample size used to make that estimate. Thus, random error disappears in an infinite sample. Systematic error is such a deviation

caused by incorrect assumptions in the estimate itself, and will not only remain, but can be increased in larger samples.

For parsimony analyses, as long as the number of changes in the sequences being compared is relatively small, then given enough data, the correct phylogeny will be reconstructed. However, when the number of changes increases to the point that there are proportionately more examples of convergent or parallel evolution (increases in homoplasy), parsimony (as well as other approaches) may be less capable of discriminating homoplastic characters. This source of systematic error is probably most serious in phylogenetic trees consisting of both long and short branches ***(20)***. To avoid or at least reduce systematic error, several things can be done. Character weighting (such as differentiating between transversions and transitions as mentioned above) is routinely performed. The elimination of long branches that reflect large divergences can be difficult, but the inclusion of multiple outgroups (which have shared primitive characters) can often diminish these effects. In addition, if there are questions about positional homology, removal of these characters can reduce the problem. Finally, changing the assumptions of the analysis can also diminish systematic error.

From a practical perspective, random error affects all phylogenetic studies, since it can only be eliminated if one collects an infinite amount of data. This unrealistic approach to research can be circumvented in large part by maximizing the extraction of the phylogenetic information by using the most appropriate methods. It is also advisable to use methods that can estimate the sensitivity of the results given the number of samples that are available. Several approaches are useful toward this end: Two of the most commonly applied methods are included here.

3.4.1. Evaluating Hierarchical Structure

The removal of all random covariation in any data set is practically impossible. However, such information constitutes noise and can even lead some phylogenetic methods to choose one tree topology instead of another, although there is no real hierarchical structure in the data to support such a choice. Therefore, it is important to be able to evaluate if there is more hierarchical structure to a data set than would be expected by chance.

Permutation tests are one way of testing for hierarchical structure. From a phylogenetic perspective, they permute the data set by randomizing character states among taxa (or sequences); simultaneously they hold the number of occurrences of any particular character-state constant, which destroys any possible correlation among character-states resulting from phylogenetic signal. If a test statistic from the permuted data set is tested with a null hypothesis generated from a number of permuted data sets, then one can determine whether the

null hypothesis of no phylogenetic structure is supported. If the test statistic for the data set being evaluated does not lie in one of the tails (5% level) of the null distribution, then there is a good chance that it arose in the absence of meaningful hierarchical structure ***(5)***.

Another way to test hierarchical structure in a data set is by evaluating the shape of the distribution of all possible trees (or at least a random sample of them). Hillis and Hulsenbeck ***(21)*** showed that as the amount of hierarchical structure in a data set increased, the distribution of tree lengths became more left-skewed, and concomitantly that data sets with little hierarchical structure produced more symmetrical tree-length distributions. The amount of skewness can be quantified using the g_1 statistic. When calculated, if the g_1 statistic is a negative number generally less than –0.5, there is considerable hierarchical structure to the data set.

3.4.2. Individual Branch Support: Bootstrap Analysis and Bremer Support Index

The methods for evaluation of random error discussed above deal primarily with the entire data set and are used to determine whether there is actually a phylogenetic signal or just random noise. As Hillis et al. ***(22)*** point out, "These approaches are designed with hypothesis-generating (rather than hypothesis testing) studies in mind." In other words, there is no previous hypothesis that is being tested, a reliable estimate for the phylogeny of the group is what is being tested. How can the reliability of the reconstructed branches be determined? One of a series of resampling methods, Bootstrap analysis ***(23)***, resamples data points with replacement to form pseudoreplicates of the data set. When one starts with a recovered topology (i.e., an *a priori* hypothesis), the relative number of times that a certain branch is recovered can be ascertained and the support for that branch presented on the tree (generally shown as a percentage). It is advisable to run at least 1000 bootstrap replicates (*see* **ref. *24***, for typical steroid hormone receptor analysis). The bootstrap value should be at least 85% to presume strong support for a branch.

Another approach to the problem of evaluating a branch (or a node) is to use the difference in tree lengths between the shortest trees that contain the monophyletic group that is represented on the branch versus those that do not contain the group. This assessment is called the Bremer Support (or sometimes referred to as the "Decay") Index ***(25)***. For molecular sequence data, this calculation is essentially the number of sequence changes that must occur for a branch to disappear. The greater the number, the higher the level of support for the node and resulting branches. In studies to date, it appears empirically that decay numbers of 10 or higher suggest reasonable support for a node. There is no absolute correlation between the bootstrap value and the Bremer Support

Index probably because of the different ways that these two measures of support are estimated. Thus, many authors choose to use both estimates.

4. Notes

1. Although both are representations of phylogenetic hypotheses, a cladogram is a branching diagram of relationships only, a tree emphasizing the pattern of evolution. Branch length is meaningless in a cladogram. In a phylogram, the branch lengths are proportional to the amount of evolutionary change that has occurred.
2. Most methods of phylogenetic analysis generate an unrooted tree unless directed to do differently. "Unrooted" simply refers to a tree in which the earliest point in time (the location of the common ancestor) is not identified. Outgroup analysis allows a tree to be rooted, based on the taxon (or sequence) that shared a common ancestor with a member of the ingroup most recently. The use of an outgroup taxon is generally advised.
3. Strict transversion parsimony is relatively harsh approach, carrying the presumption that there is little or no valuable information in transitions. Over long periods of divergence, there can be saturation of transitions with respect to transversions, but for recently diverged taxa (or genes), transitions can still retain a great deal of information. Thus, in many cases researchers differentially weight transversions over transitions (while these weights can be calculated in a number of ways, many researchers feel they are best estimated from the ratio of transitions to transversions present in the data set being evaluated).
4. Wagner parsimony is similar to Fitch parsimony, except that the Wagner method allows minimal constraints on character-state changes; the Fitch method allows no such constraints. Possibly the major constraint is that Wagner parsimony assumes interval data, and therefore is highly appropriate for binary and ordered multistate characters (not common in nucleotide or amino acid sequence data sets).
5. In some cases, this method (MGC parsimony) may be considered computational overkill because it pays strong attention to third-position (silent) substitutions that do not cause amino acid changes.
6. Swofford et al. ***(5)*** conclude that the computations required for the general parsimony algorithms in PROTOPARS are simplified with respect to MGC parsimony, because all potential codons that are translated into a particular amino acid are not considered nor are all of the potential synonymous codon assignments to interior nodes.
7. Heuristic methods do not always find the most optimal tree topology. They are limited by the starting tree that is being rearranged and by the order that taxa (or sequences) are added.

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II

Purification Protocols

3

Expression and Purification of Recombinant Human Progesterone Receptor in Baculovirus and Bacterial Systems

Vida Senkus Melvin and Dean P. Edwards

1. Introduction

1.1. Full-Length Progesterone Receptor in Baculovirus System

Human progesterone receptor (PR) is a member of the nuclear hormone receptor superfamily of transcriptional activators, which share a common modular structure consisting of a C-terminal ligand-binding domain (LBD), a highly conserved and centrally located DNA-binding domain (DBD), and a poorly characterized N-terminal domain that is required for maximal transcriptional activity ***(1,2)***. Human PR is expressed as two proteins from a single gene by alternate use of two promoters: PR-A, which is missing the first 164 amino acids in the N-terminus, and full-length PR-B ***(3)***.

As with other transcription factors, nuclear hormone receptors are expressed in target tissues and cells in low amounts, which necessitates the development of recombinant protein expression systems to conveniently produce and purify receptors for subsequent use in biochemical and structural studies. Recombinant systems are also of value for production and analysis of various receptor domains. Bacterial systems have been useful for expression of the DBD and LBD of several different nuclear receptors including human PR ***(4)***. However, bacteria have not generally been useful for expression of full-length nuclear receptors, probably because of lack of post-translational modification(s) and folding of these large and complex mammalian proteins, resulting in their aggregation and/or proteolysis. The baculovirus insect expression system has generally been more useful for production of full-length nuclear receptors in a biologically active form ***(5,6)***. Eukaryotic insect cells have been found to cor-

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rectly fold and post-translationally modify many foreign expressed mammalian proteins. Indeed, the authors, and others, ***(7–12),*** have found that full-length human PR expressed in *Spodoptera frugiperda* (Sf9) insect cells exhibits functional properties similar to that of native PR in breast cancer cells including steroid binding, DNA binding, and transcriptional activity in vitro. The authors also reported that human PR expressed in Sf9 insect cells is correctly phosphorylated on the same serine residues as endogenous PR in mammalian cells ***(13)***. This chapter describes the production and purification of full-length PR in the baculovirus insect cell system and the DBD of PR in a bacterial system.

There are a number of excellent commercial transfer plasmids with convenient restriction sites for constructing recombinant baculoviruses. A summary of transfer plasmids and methods for construction of recombinant baculoviruses is beyond the scope of this chapter, but can be found in other volumes and reviews ***(14–16)***. To express PR as a nontagged protein using its own ATG translation start site, PR-A and PR-B cDNAs were cloned into the baculovirus transfer plasmid, pVL1392 (Pharmingen) ***(8)***. A histidine (His)-tagged PR was constructed by inserting PR-A and PR-B cDNAs into pBlueBacHis2 (Invitrogen), which places the PR coding sequences in frame at the N-terminus with plasmid sequences containing an ATG translation start site, six consecutive His residues, and an enterokinase cleavage site ***(17)***. Described are the purification of nontagged PR by monoclonal antibody (MAb) affinity chromatography and the purification of His-tagged PR by nickel affinity resins.

MAb purification has the advantage of providing highly purified (>95%) receptors as a single step procedure. It also does not require use of sequence tags that could potentially alter the functional properties of PR or interfere with subsequent applications. The disadvantages of MAb purification are that it requires denaturation for PR elution from the resins, the yields are generally lower than with His-tagged PR on nickel resins and the construction of MAb affinity resins is expensive and more involved than purchase of relatively inexpensive nickel resins. However, the cost of MAb columns can be minimized by multiple reuse. With appropriate regeneration procedures, MAb columns can be reused up to 15 times before degradation of the resins occurs. Thus, MAb purifications are useful for obtaining smaller amounts of highly purified PR as a nonfusion protein. Approximately 0.5 mg of PR is typically purified from a 500 mL Sf9 cell culture at a concentration of 100–200 μg/mL. This is a useful amount for DNA binding, transcription assays, as a kinase substrate and for other biochemical applications.

The advantages of His-tagged PR are the convenience and ease of nickel affinity purification, the low cost of nickel resins, and the ability to elute PR

from the resins under nondenaturing conditions by competition with imidazole. Because of the high affinity of nickel resins for 6X histidine residues, the % binding and yield of purified product is generally higher than MAb purification. This also offers a universal method for purification of expressed PR domains engineered to have 6X His-tags. Indeed, the authors have expressed various domains of PR with N-terminal poly-histidine tags and using methods similar to those described here have obtained highly purified PR fragments for in vitro biochemical experiments ***(10,18)***. A disadvantage is nonspecific protein binding to nickel resins, which generally results in a lower degree of purity than purification by MAb columns necessitating a prior or subsequent purification step(s). Including DNA cellulose as a second step increases the level of purity to ≥ 90% and also selects for functional receptor. Another potential disadvantage of tagged PR is that the poly-histidine tag alters the functional properties of PR, or interferes with subsequent applications, such as protein–protein interaction with another His-tagged protein by nickel resin pull down assay ***(10,18)***. However, purified nonfusion PR by MAb columns and His-PR by nickel-resin were found to have indistinguishable DNA binding activity ***(9,17)***. Some baculovirus vectors, such as the pBlueBacHis2 used here, contain an enterokinase cleavage site for removal of the poly-histidine tag. The authors have found that enterokinase cleavage in general is not quantitative and the efficiency of cleavage can vary a great deal depending on the fusion protein sequence ***(10,18)***.

Purified PR obtained by either approach is biologically active and useful for in vitro DNA binding and transcription assays. In the presence of appropriate coregulatory proteins, purified PR binds with high affinity to specific target DNA sequences by electrophoretic gel mobility shift assay ***(9,17)*** and stimulates rates of transcription from appropriate DNA templates containing specific PR binding sites ***(11)***. The fraction of purified PR that is biologically active has not been carefully documented. By radioligand exchange binding assay, greater than 60% of purified PR protein could be accounted for as having steroid binding activity, suggesting that the majority, but not all, purified product is biologically active. The fraction of purified PR protein that exhibits DNA binding and transcriptional activity has not been estimated. Purification of unliganded PR by the above procedures results in lower yields than liganded PR and the product exhibits little steroid binding activity. Thus, unliganded PR is functionally unstable in vitro. Methods have not been attempted to refold and renature unliganded full-length PR. Purified PR bound to R5020 can be used for steroid binding in a limited way by radioligand exchange assay. Incubation at 4°C with ^{3}H-R5020 results in some exchange between unlabeled and ^{3}H-R5020 bound to PR.

1.2. Purification of Steroid Receptor DBD Expressed in Bacterial Cells

Bacterial expression systems have been used extensively for production and purification of various nuclear receptor DBDs (***19–25***, and references therein). Although large-scale expression and purification of the DBD can be achieved in the baculovirus system described above ***(26)***, bacterial expression is faster, because it does not require lengthy transfection procedures or homologous recombination, and bacterial cell culture and maintenance is cheaper. Furthermore, the DBD fragment, unlike full-length receptors, does not appear to require post-translational modifications for its function and therefore does not require an eukaryotic expression system. Finally, bacteria efficiently produce high concentrations of biologically active DBD fragments for use in both biochemical and structural studies.

Efficient bacterial expression of any protein requires the combination of protease-deficient bacterial strains and an inducible expression system. BL21 cells (Pharmacia) are often used in recombinant protein production because they are deficient in the *lon* gene, encoding the major bacterial protease, as well as the *ompT* gene, coding for an outer membrane protease, both of which are responsible for most recombinant protein cleavage ***(27–29)***. Use of BL21 cells greatly increases the yield of fusion protein from bacterial cell lysates and reduces the risk of purification of degradation products. In order to avoid deleterious effects of foreign protein overexpression, most bacterial expression systems use inducible promoters to drive recombinant protein expression. This lab and others use the pGEX expression system (Pharmacia), which utilizes the Ptac promoter to drive recombinant protein expression. The Ptac promoter is under the control of the lac repressor present in most bacterial strains. Isopropyl-β-D-thiogalactoside (IPTG) inhibits binding by the lac repressor; therefore, addition of IPTG to bacterial cultures induces recombinant protein expression from the Ptac promoter. pGEX vectors produce recombinant proteins as fusions with glutathione-*S*-transferase (GST), which has high affinity for glutathione, and purification of the GST-fusion protein is a simple one-step method using glutathione-bound beads. Elution of the immobilized fusion protein is achieved under nondenaturing conditions by competition with soluble glutathione. pGEX vectors also encode a thrombin or factor Xa cleavage site between the GST-tag and the recombinant protein for easy removal of the GST-tag, leaving a free, untagged DBD. The last step in purification requires separation of the free DBD and the GST-tag on DNA cellulose. This step also ensures that the purified DBD maintains its high affinity for DNA, since only those DBDs capable of binding DNA are purified. The three-step expression and purification procedure described in **Subheading 3.3.** yields highly purified, concen-

trated DBDs with DNA-binding activity identical to DBDs purified from eukaryotic systems, such as baculovirus (***26***; Melvin and Edwards, unpublished data). A 1.0 L bacterial culture yields approx 0.5–1.0 mg total protein at ~90% purity, which is sufficient for biochemical analysis. This purified PR DBD, in the presence of appropriate coregulatory proteins, has high affinity for its target DNA element as judged by electrophoretic mobility shift assays (EMSA, k_d = 15–20 n*M* (***26***; Melvin and Edwards, unpublished data). Increasing the volume of cultures and further concentration steps should yield sufficient protein for structural analysis. Protocols for cloning into expression plasmids, or maintenance of bacterial strains are beyond the scope of this chapter, and can be obtained in other molecular biology methods manuals.

2. Materials

2.1. Full-Length PR in the Baculovirus System

2.1.1. Insect Cells

1. *Spodoptera frugiperda* (Sf9) insect cells are grown in Grace's insect medium (Gibco-Life Sciences) supplemented with 3.3 g/L yeastolate (Difco), 3.3 g/L of lactalbumin hydrolysate (Difco), 10% heat-inactivated fetal bovine serum (Hyclone labs) and 50 μg/mL of Gentamicin (Gibco). For cells grown in bioreactors, 0.1% Pluronic F68 is added (*see* **Note 1**).
2. Culture vessels for Sf9 cells: For small scale cultures up to 500 mL, cells are grown in suspension in conventional spinner vessels with constant stirring (Bellco Glass). For large-scale cultures, cells are grown in a 5 L bioreactor (Applikon, Inc.) maintained at 50% saturated oxygen with constant sparging and stirring ***(15)***.
3. Recombinant PR baculovirus.

2.1.2. Nontagged PR

1. Cell lysis/TEDG buffer: 10 m*M* Tris-base, pH 7.4, 1 m*M* ethylenediamine tetraacetic acid (EDTA), 1 m*M* dithiothreitol (DTT), 10% glycerol, 400 m*M* NaCl and 1X protease inhibitors.
2. 100X Protease inhibitors: 50 μg/mL leupeptin, 1 mg/mL bacitracin, 200 μg/mL aprotinin, and 100 μg/mL pepstatin (Sigma).
3. MAb affinity resin: MAb AB-52 that recognizes the A and B forms of human PR, or B-30, which reacts with PR-B only ***(30)***, were chemically crosslinked to protein G Sepharose (Pharmacia) with 10 m*M* dimethylpimilimidate (Pierce) at a substitution of 6–8 mg/mL beads as previously described ***(9)***. Resins are stored at 4°C in TEG buffer (10 m*M* Tris-base, pH 7.4, 1 m*M* EDTA, 10% glycerol) containing 0.02% sodium azide. Approximately 1 mL MAb resin is used to purify PR from each 300–500 mL Sf9 cell culture. Just prior to use, MAb resins are washed three times in 10 mL TEG.

4. MAb-coupling reagents: 0.1 *M* borate coupling buffer, pH 8.2, 0.2 *M* triethanolamine, pH 8.2, 20 m*M* dimethylpimilimidate (prepared fresh before use in 0.2 *M* triethanolamine, pH 8.2), 20 m*M* ethanolamine.
5. MAb resin elution buffer: 50 m*M* Tris-base, 1 m*M* EDTA, 20% glycerol adjusted to pH 11.3 with 1 *N* NaOH.
6. PR renaturation/neutralization buffer: 400 m*M* Tris-HCl, pH 7.4, 40 m*M* $MgCl_2$, 40 m*M* DTT, 4 m*M* EDTA, 0.4 m*M* EGTA, 100 m*M* NaCl, 0.2 m*M* $ZnCl_2$, and 50% glycerol.
7. Regeneration of MAb resins: Resins are washed sequentially in 10 mL of the following buffers: Twice in TEG, once in TEG plus 1 *M* NaCl, once in TEG, once in 1 *M* sodium thiocyanate, three times in TEG. Store regenerated beads in TEG plus 0.02% sodium azide at 4°C.

2.1.3. His-Tagged Full-Length PR

1. Cell lysis buffer: 20 m*M* Tris-HCl, pH 8.0, 350 m*M* NaCl, 5 m*M* imidazole, 10% glycerol, 15 m*M* β-mercaptoethanol and 1X protease inhibitors (*see* **Note 2**).
2. Nickel affinity resin (Ni-NTA Agarose, Qiagen). Resins are prepared by washing three times in 40 mL with cell lysis buffer lacking the protease inhibitors. Beads should be prepared fresh just before use. Approximately 2 mL of packed beads are used to purify His-tagged PR from 500 mL of Sf9 cell cultures.
3. High-salt wash buffer: 20 m*M* Tris-HCl, pH 8.0, 600 m*M* NaCl, 5 m*M* imidazole, 10% glycerol, 15 m*M* β-mercaptoethanol (*see* **Notes 2** and **3**).
4. Low-salt wash buffer: 20 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 5 m*M* imidazole, 10% glycerol, 15 m*M* β-mercaptoethanol (*see* **Notes 2** and **3**).
5. Ni-NTA resin elution buffer: 20 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 250 m*M* imidazole, 10% glycerol, 15 m*M* β-mercaptoethanol.
6. Purified, His-tagged PR storage buffer: To stabilize the biological activity of purified PR, 1000X DTT (final 1 m*M*), 1000X $ZnCl_2$ (final 1 μ*M*), and 1000X EDTA (final 1 m*M*) are added to PR eluted from the Ni-NTA resins.
7. DNA cellulose (native double stranded calf-thymus DNA cellulose; Amersham Pharmacia Biotech). Prewash DNA cellulose fresh before use in 20 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 10% glycerol, 1 m*M* DTT, 1 μ*M* $ZnCl_2$ and 1 m*M* $MgCl_2$. Use approx 1 mL of packed DNA cellulose per 500 mL of Sf9 cell culture.
8. DNA cellulose elution buffer: 20 m*M* Tris-HCl, pH 8.0, 400 m*M* NaCl, 10% glycerol, 1 m*M* DTT, 1 m*M* $MgCl_2$.
9. EnterokinaseMax (Invitrogen): For cleavage of the N-terminal 6X histidine tag from PR immobilized to Ni-NTA resins, wash resins in TG buffer (20 m*M* Tris-HCl, pH 8.0, 10% glycerol) and incubate purified, His-tagged PR for 16 h at 4°C with 2 U of enzyme per 1 μg of PR. Cleaved PR released from the resins is separated from enterokinase by absorption of the enzyme to soybean trypsin inhibitor affinity resin (Sigma).
10. Siliconized tubes: Tubes for storage or holding of purified PR are siliconized either by a siliconizing agent, such as Sigmacote, or presiliconized microcentrifuge tubes are purchased (S&S Scientific) (*see* **Note 4**).

2.2. PR DBD in Bacteria

1. BL21 cells expressing the fusion protein: Typically, the initial overnight culture is inoculated from a single bacterial-streak colony; however, glycerol stocks can also be used.
2. Luria broth (LB).
3. 200 m*M* IPTG: This reagent can be made ahead of time and stored at –20°C in frozen aliquots wrapped in foil.
4. 1:1 Glutathione Sepharose 4B (Amersham Pharmacia Biotch) in 1X phosphate-buffered saline (PBS): To prepare the beads, wash twice in 50 mL 1X cold PBS, then resuspend in a volume of 1X PBS equivalent to the bead bed volume. The prepared beads can be stored in this 1:1 suspension for up to 1 mo at 4°C.
5. 1 *M* Glutathione (100X): 1 *M* stock is prepared in 50 m*M* Tris, pH 8.0, aliquoted, snap-frozen in liquid nitrogen (LN_2) and stored at –80°C for several months. At 1 *M*, glutathione does not go readily into solution, so pipet or vortex this slurry to mix prior to addition to buffers. Additionally, the 100X stock should be diluted in the glutathione Sepharose elution buffer immediately before use to maintain stability.
6. Thrombin: Thrombin is resuspended in 1X filter sterilized, cold PBS at a concentration of 1 cleavage unit/μL (1 cleavage unit = 0.2 NIH units). Aliquot, freeze in LN_2, and store at –80°C in siliconized microfuge tubes. This solution is sensitive to repeated freeze–thaw cycles, so it should be stored in small aliquots.
7. 1X PBS: 140 m*M* NaCl, 3 m*M* KCl, 8 m*M* Na_2PO_4, 2 m*M* KH_2PO_4, pH 7.4.
8. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 250 m*M* KCl, 1% Triton X-100, 5 m*M* DTT, 5 μ*M* $ZnCl_2$ (*see* **Note 5**).
9. Glutathione Sepharose elution buffer: 50 m*M* Tris-HCl, pH 8.0, 2.5 m*M* $CaCl_2$, 50 m*M* KCl, 1 m*M* DTT, 50 μ*M* $ZnCl_2$, 10% glycerol, 10 m*M* glutathione (*see* **Note 6**).
10. DNA cellulose buffer: 20 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 1 m*M* DTT, 50 μ*M* $ZnCl_2$, 10% glycerol.

3. Methods

3.1. MAb Purification of Nontagged Full-Length PR

1. Preparation of MAb beads. Purified MAbs are dialyzed against the borate-coupling buffer and incubated in batch with protein G Sepharose (Amersham Pharmacia Biotech) for 30 min at room temperature (RT) at a ratio of 8–10 mg MAb/mL beads. The volume of MAb to beads should not exceed 4:1 (vol:vol, MAb:packed beads). Beads are washed twice in coupling buffer, twice in triethanolamine, resuspended in 20 vol dimethylpimilimidate crosslinker, and incubated in batch for 45 min at RT. Collect beads by centrifugation, and save flowthrough. Deactivate by incubating beads in suspension with 20 vol ethanolamine for 30 min at RT, then wash beads three times in borate-coupling buffer, three times in TEG and store at 4°C in TEG plus 0.02% sodium azide preservative. To determine the extent of MAb crosslinking, measure the starting MAb

solution, flowthrough, and wash fractions for protein concentration by Bradford assay and by sodium dodecyl sulfate (SDS) gel electrophoresis and Coomassie blue staining.

2. Sf9 cells are plated in suspension cultures at a density of 10^6 cells/mL in 500 mL spinner vessels and infected with recombinant baculovirus at MOI 1.0 for 48 h at 27°C (*see* **Note 7**). The synthetic progestin R5020 at 200 n*M* is added to cultures for the last 24 h of expression (*see* **Note 8**).
3. Harvest Sf9 cells and lyse in 5X vol (cell pellet:lysis buffer) TEDG buffer plus 0.4 *M* NaCl. Cells are lysed by 10–15 strokes in a Potter-Elevehjem tissue grinder. Centrifuge lysates at 100,000*g* for 30 min and collect the supernatant fraction, which contains soluble PR. The supernatant is dialyzed against TEDG to reduce the salt concentration (*see* **Note 9**).
4. Dialyzed supernatants are incubated for 4 h at 4°C as a suspension with 1 mL MAb resins on an end-over-end rotator. Approximately 1 mL of MAb beads is used to purify PR from each 500 mL Sf9 cell culture. Collect beads by centrifugation for 5 min at 1500 rpm and save the flowthrough fraction. Beads are washed in batch by resuspension in excess (10–15 mL) wash buffer (TEG), pelleting of the beads by centrifugation at 1500 rpm × 5 min and discarding the supernatant. The wash step is repeated three times with TEG containing 0.4 *M* NaCl, followed by a single wash in TEG.
5. Transfer washed MAb beads to a new siliconized 15-mL conical tube and wash once more with TEG (*see* **Note 10**).
6. To elute bound PR, beads are exposed to alkaline pH by resuspension in 600 µL pH 11.3 elution buffer at 4°C. The beads are immediately pelleted by centrifugation at 1500 rpm × 5 min. The supernatant with released PR is transferred to a clean, siliconized tube, and the alkaline pH elution step is repeated four times, combining the supernatants of each elution together.
7. The pooled eluants are immediately neutralized by addition of the renaturation/neutralization buffer. This brings the pH of eluted PR back to 7.4.
8. Aliquot the purified PR in siliconized microcentrifuge tubes in convenient sizes, snap freeze in liquid nitrogen, and store at –80°C.
9. Analyze the purity of PR by SDS-polyacrylamide (7.5%) gel electrophoresis and silver staining, and confirm the identity of the protein by Western blot (**Fig. 1** shows single step purification of PR-A and PR-B by MAb affinity columns). To determine the protein concentration of purified PR, quantitative silver-stained SDS-gel electrophoresis is recommended. Bradford and Lowry assays tend to overestimate PR concentration by as much as threefold. To quantitate PR by silver-stained gels, electrophorese varying known amounts of purified bovine serum albumin (0.25–1 µg) with different unknown amounts of PR. Compare the intensities of stained bands by densitometric scanning, or by visualization.

3.2. Purification of His-Tagged PR

1. Expression in Sf9 cells is the same as above (*see* **Subheading 3.1.**), except that scale-up production in 5 L bioreactors is more typically performed with His-tagged PR, because of the lower expense of nickel-affinity resins, compared with

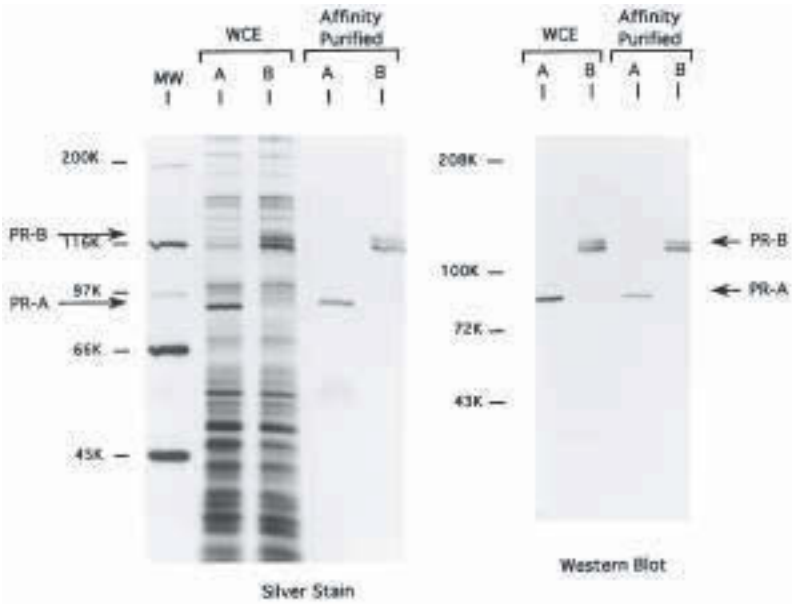

Fig. 1. Purification of recombinant PR-A and PR-B by MAb affinity chromatography. WCEs were prepared from Sf9 cells after infection with recombinant baculovirus vectors expressing either the A or B isoform of human PR. To bind receptor to hormone in vivo, cells were incubated with R5020 for 4 h, just prior to harvest. Receptors were purified from WCEs by MAb affinity chromatography using B-30 for purification of PR-B and AB-52 for purification of PR-A. WCEs and purified products were analyzed by silver-stained SDS-gels (A) and by immunoblotting with AB-52 (B). MW, molecular weight standards. Reprinted with permission from **ref. *9***.

MAb resins, for downstream purification of receptors. Cells are grown in a bioreactor to a density of 1.5–1.6 × 10^6/mL, then inoculated with virus at an MOI of 1.0. Cells are grown for an additional 32–36 h at 27°C and typically reach a density of 2 to 2.2 × 10^6 cells/mL at the time of harvest.

2. Large-scale purification is a two-step procedure involving Ni-NTA resins used in a batch/column manner followed by DNA cellulose. Smaller-scale single-step batch purification of His-tagged PR on nickel reins from 500 mL spinner cultures can also be performed.
3. Cells from a 5 L bioreactor are harvested and processed in 10 × 500 mL pellets. Each pellet is lysed as in **Subheading 3.1.** in 20 mL His-tagged PR lysis buffer. The lysates are combined from the 10 pellets and centrifuged at 12,500*g* for 30 min. The supernatant is then recentrifuged at 100,000*g* for 60 min, and this high-speed supernatant (termed "whole-cell extract" [WCE]) containing soluble His-tagged PR is collected (*see* **Note 9**).
4. Total protein concentration of the supernatant is measured by Bradford assay and the volume of the supernatant diluted with lysis buffer, if necessary, so that

the protein concentration does not exceed 12 mg/mL. Concentrations higher than 12 mg/mL tend to increase nonspecific protein binding to the Ni-NTA resin. The total volume of the cell lysate should be 250–300 mL.

5. Approximately 10 mL (packed volume) of prewashed Ni-NTA resins are divided into 1 mL aliquots in 50 mL siliconized, plastic centrifuge tubes. Each 1 mL Ni-NTA resin is incubated with 30 mL WCE as a suspension for 1 h at 4°C on an end-over-end rotator. Beads are then pelleted by centrifugation at 1500 rpm for 5 min and the flowthrough fraction saved.
6. The collected Ni-NTA resin is each washed separately in the same 50 mL conical tubes by resuspension in 45 mL of high-salt wash buffer, centrifugation, and discarding of the supernatant (*see* **Note 3**). The washed beads are combined into a single 50 mL conical and washed three more times.
7. The combined Ni-NTA resin is then transferred to a siliconized glass column (1.5 × 10 cm Bio-Rad Econocolumn), packed under gravity with low-salt wash buffer, and the flowthrough connected to a 280 nm UV monitor and automatic fraction collector (Pharmacia). The column is washed until the optical density decreases to the baseline buffer value.
8. Bound, His-tagged PR is eluted by competition under nondenaturing conditions by 250 m*M* imidazole, which structurally resembles His. Elution buffer is passed over the column at a flow rate of 1–2 mL/min, and 1 mL fractions are collected into siliconized tubes. The eluted protein peak fractions are detected by UV absorbance and pooled.
9. The pooled eluate is immediately incubated in siliconized tubes on an end-over-end rotator with approx 1 mL (packed volume) prewashed DNA cellulose for 30 min at 4°C. The DNA cellulose is washed by repeated resuspension and centrifugation in wash buffer and eluted in batch by resuspension in buffer containing 0.4 *M* NaCl. Resuspended resin in 1 mL elution buffer is incubated on an end-over-end rotator for 10 min at 4°C, and the supernatant with eluted PR collected by centrifugation at 1500 rpm × 5 min. This elution step is repeated and the two supernatants are combined.
10. Aliquot purified PR into siliconized microcentrifuge tubes, snap freeze, and store at –80°C (*see* **Note 4**). Samples are analyzed for purity and PR concentration as above (*see* **Subheading 3.1.** and **Note 11**).

3.3. Purification of the PR DBD

3.3.1. Culture of Bacterial Cells, Induction of Recombinant DBD Expression, and Preparation of Cell Lysate

1. Inoculate a 10 mL LB + 50 μg/mL ampicillin culture from BL21 cells expressing the GST DBD. Incubate shaking overnight at 37°C.
2. Dilute the culture into a 2.0 L culture flask containing 500 mL LB + 50 μg/mL ampicillin and incubate at 37°C shaking.
3. When absorbance at λ = 600 n*M* reads 0.8, induce expression of fusion protein by addition of 200 m*M* IPTG to 0.5 m*M* final concentration (1.25 mL in 500 mL culture). Incubate shaking for an additional 3 h at 37°C (*see* **Note 12**).

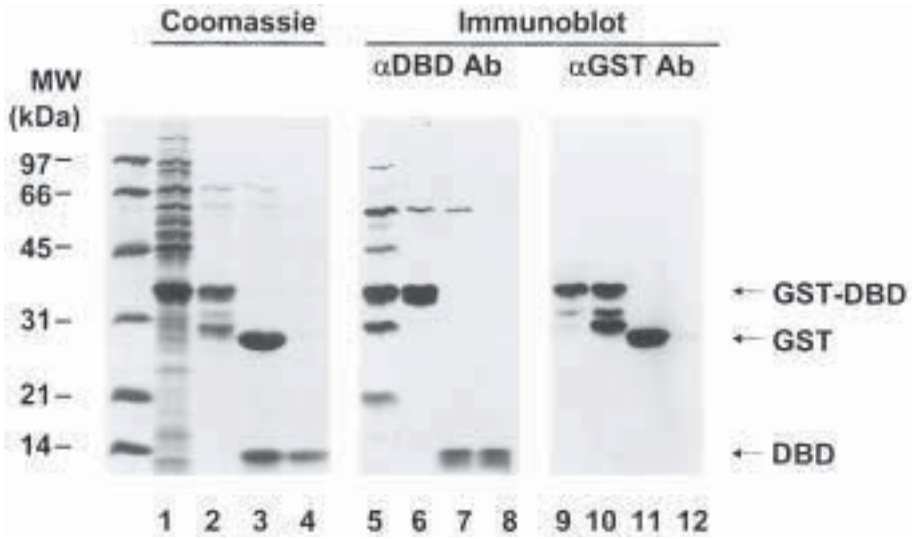

Fig. 2. Purification of recombinant PR DBD from bacterial cells. Bacterial cell lysates were prepared from cells expressing GST-PR DBD fusion protein (*lanes 1*, *5*, and *9*). The intact GST-PR DBD bound and eluted from glutathione Sepharose (*lanes 2*, *6*, and *10*). Thrombin-cleaved GST-PR DBD (*lanes 3*, *7*, and *11*), and the free DBD purified on DNA cellulose (*lanes 4*, *8*, and *12*). WCEs and purified products were analyzed by a Coomassie-stained SDS gel (*lanes 1–4*) or immunoblot with antibodies recognizing the PR DBD (*lanes 5–8*) or the GST-tag (*lanes 9–12*). MW, molecular weight standards.

4. Harvest bacterial cells by centrifugation at 5000*g* for 10 min at 4°C. Wash the cells by resuspending in 25 mL in ice-cold 1X PBS and pellet again. Discard the supernatant.
5. Resuspend the cell pellet in 25 mL lysis buffer plus 1X protease inhibitor cocktail. Ensure that the pellet is completely resuspended and lyse by two freeze–thaw cycles in liquid nitrogen at 37°C (*see* **Note 13**).
6. Once thawed, clarify the lysate by centrifugation for 30 min at 100,000*g* at 4°C. (**Figure 2**, lanes 1, 5, and 9 show Coomassie-stained SDS gel and Western blots of bacterial cell lysate; *see* **Note 14**.)

3.3.2. Purification of GST DBD

Note: The remainder of the procedures should be performed on ice or at 4°C.

1. Incubate the bacterial cell lysate with 2.0 mL of 1:1 suspension of glutathione Sepharose beads in 1X PBS (1.0 mL packed beads) on an end-over-end rotator for 2 h in a 50 mL siliconized conical at 4°C (*see* **Note 5**).
2. Pellet the beads by centrifugation at 1500 rpm and wash the GST DBD-bound glutathione beads twice in 50 mL cold 1X PBS. Transfer the 1.0-mL bead

volume to a 2.0 mL siliconized microfuge tube and wash in 1.0 mL cold 1X PBS. Pellet the beads again.

3. Elute the glutathione-bound fusion protein by competition with 1.0 mL glutathione Sepharose elution buffer. Incubate on an end-over-end rotator for 10 min at 4°C. Pellet the resin by centrifugation at 1500 rpm for 5 min, collect the supernatant, and repeat elution procedure for a total of three elutions. (**Figure 2**, lanes 2, 6, and 10 show eluted GST DBD; *see* **Note 6**.)
4. Determine the protein concentration of purified GST DBD using a Bradford assay (Bio-Rad, per manufacturer's instruction). Add 10 U thrombin/mg GST-fusion protein. Incubate on an end-over-end rotator at 4°C for 10–16 h. (**Figure 2**, lanes 3, 7, and 11 show post-thrombin cleavage reaction; *see* **Note 15**.)
5. Prepare three microfuge tubes, each containing 0.25 mL bed volume of DNA cellulose. Wash three times in 1.0 mL DNA cellulose buffer.
6. Divide the thrombin-treated GST DBD preparations into three aliquots and incubate each with the DNA cellulose on an end-over-end rotator for 30 min at 4°C.
7. Pellet the DNA cellulose by centrifugation at 1500 rpm. Wash the DNA cellulose three times in 1.0 mL DNA cellulose buffer. On the last wash, pool the three aliquots of DNA cellulose into a single 2.0 mL siliconized microfuge tube and centrifuge at 1500 rpm and discard the supernatant.
8. Elute the DBD by resuspension of DNA cellulose in 1.0 mL DNA cellulose buffer + 450 m*M* NaCl (500 m*M* NaCl final). Rotate for 10 min at 4°C. Pellet the resin by centrifugation at 1500 rpm for 5 min, collect the supernatant, and repeat for a total of two elutions.
9. Combine the two eluates and dialyze the purified DBD in 2.0 L DNA cellulose buffer to reduce salt, which interferes with DNA binding. (**Figure 2**, lanes 4, 8, and 12 show purified DBD free of the GST-tag.)
10. Aliquot the purified DBD into siliconized microfuge tubes and snap freeze in liquid nitrogen. Store at –80°C.

4. Notes

1. There are several insect cell lines available for production of recombinant proteins from baculoviruses. In comparing three cell lines, the authors found that Sf9 cells were slightly better for PR production than Sf21, and much better than the high 5 (Trichoplusia ni) cells ***(6)***.
2. A low concentration of imidazole is included in the cell lysis buffer and wash buffer to minimize nonspecific protein binding to the nickel resin. The differential affinity of nonspecific proteins and the 6X His-tagged PR for the Ni-NTA resin is large enough that 5–10 m*M* imidazole does not interfere with PR binding, but does disrupt nonspecific protein binding.
3. The high-salt wash buffer reduces nonspecific binding of cellular proteins to the Ni-NTA resin. The resin is then washed in a low-salt wash buffer to reduce salt concentrations for the later purification steps on DNA cellulose.
4. Purified PR has a propensity to bind to the walls of plastic tubes. To minimize loss of PR, all tubes and columns used in the purification and storage of receptors

should be siliconized. Purified PR at –80°C is stable in terms of DNA binding for 3–4 mo, as long as samples are not repeatedly frozen and thawed. Therefore, receptor should be stored in aliquot sizes that are expected to be used for different applications.

5. Triton X-100 and high concentrations (5 m*M*) of DTT increase binding of the fusion protein to glutathione resin, as well as maintain the DBD in a chemically reduced state ***(29)***. $ZnCl_2$ is important for the structural fold of all nuclear receptor DBDs and should be included in all purification and storage buffers (with the exception of PBS washes).
6. Buffer conditions such as variations in pH, ionic strength, and glutathione concentration can affect elution of the GST DBD from glutathione Sepharose ***(29)***. If glutathione concentration is changed for elution, buffering conditions should be increased, and the pH of the elution buffer should be checked after glutathione addition because it can dramatically change the pH of the buffers.
7. The authors have optimized insect cell culture and viral infection conditions for maximal production of PR with minimal protein degradation. Little difference in PR expression has been observed by varying the MOI of viral infection between 1.0 and 10.0. Therefore, an MOI of 1.0 is typically used to save on virus. PR protein expression is not detected until 24 h after viral infection and is optimal in conventional spinners at 48 h, and in bioreactors at 32–36 h. Although longer times of viral infection give more total PR, more degradation products are also generated.
8. The addition of hormone to Sf9 cultures during the last 24 h of infection increases the total yield of PR ***(8)***. The mechanism for the increase is not known, but is probably caused by the stabilizing effect of the ligand against misfolding and degradation of the overexpressed, foreign protein.
9. Purified PR is unstable at elevated temperatures, even for short periods of time. Therefore, all steps in the purification procedure should be carried out at 4°C.
10. Transferring beads to a new tube eliminates the extraction of proteins bound nonspecifically to the wall of the tubes during incubation of the crude cell extracts.
11. From a 5 L bioreactor, we obtain on the order of 3–6 mg of purified PR at concentrations of 200–400 μg/mL. These amounts are useful for both biochemical and structural studies.
12. In bacteria, expression conditions will vary from plasmid to plasmid and even among bacterial isolates containing the same plasmid; therefore, optimization of expression conditions is extremely important. Optimization should include an analysis of the time course for cell growth or alteration in growth temperature, cell density at time of induction, length of induction time with IPTG, concentration of IPTG, and extent of aeration during cell culture ***(29)***. Optimization of conditions can also help to alleviate problems because of insolubility of recombinant proteins, but other protocols can also be used.
13. Sonication rather than freeze-thaw has been successfully used for bacterial cell lysis, but care must be taken to use mild conditions, since long sonication times

can disrupt protein folding. Additionally, when using sonication, 1% Triton X-100 should be added afterward to prevent frothing of the lysate and subsequent denaturation of expressed proteins.

14. Denaturation methods have been successfully applied to purification of insoluble GST-fusion proteins. These protocols use urea, guanidinium HCl, or sarkosyl during lysis to extract insoluble, recombinant proteins from inclusion bodies, with subsequent renaturation by dialysis or removal of denaturants upon binding the GST-fusion protein to the glutathione matrix ***(29,31–32)***. This solubilization method is less ideal, since renaturation is required to restore protein function and loss of some activity can occur. Finally, addition of non-ionic detergents (Triton X-100) can also contribute to solubilization of fusion proteins as well as promoting binding of the GST-tag to glutathione beads.
15. The authors have found that some GST-DBDs are not efficiently cleaved by thrombin in solution, rather they must be bound to the glutathione matrix for cleavage of the GST-tag. To cleave the GST-DBD when bound to glutathione Sepharose, the resin is resuspended in 5.0 mL glutathione elution buffer without glutathione and 100 cleavage units of thrombin are added. The resins are incubated overnight on an end-over-end rotator at 4°C. Pellet the resin by centrifugation and keep the flowthrough which contains the purified DBD. The resin is then washed twice more in 5.0 mL glutathione elution buffer without glutathione for 20 min at 4°C. Pellet the resin by centrifugation after each wash and pool the supernatant with the flowthrough. Glutathione Sepharose acts as a size exclusion column for proteins less than 20×10^6 Daltons, including the nuclear receptor DBD. To avoid retention of the cleaved DBD by the resin, the thrombin cleavage reaction is performed in a large volume (5:1 buffer to bead volume) and the resins are then washed in the same volume to further extract the cleaved DBD. The cleaved DBD in the pooled flowthrough and washes is then submitted to the second step purification as above to concentrate the DBD and separate it from thrombin or free GST.

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4

High-Yield Purification of Functionally Active Glucocorticoid Receptor

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1. Introduction

The glucocorticoid receptor (GR) resides in the cytoplasm as a complex with chaperones. Upon ligand association, GR shuttles into the nucleus, and binds to its hormone response elements to control expression of particular genes *(1–7)*. Transcriptional regulation by GR is thought to be a consequence of macromolecular assembly formation with proteins, including chromatin remodelers, histone modifiers, coactivators, and transcriptional machinery on a promoter *(1,8–17)*. Nucleation of this large multisubunit complex by GR homodimers results in a chromatin transition *(18–23)* and preinitiation complex assembly. To date, the molecular details of the GR-induced macromolecular assembly and chromatin transition are poorly understood.

An elucidation of the structural details of steroid receptors, as well as the dynamic interactions of the receptors and their cofactors on a chromatin template in vitro, necessitates large amounts of purified receptor. The isolated fraction should bind specifically to its target elements, interact with appropriate accessory proteins and transcription factors, and ultimately activate transcription. Chromatographic purification of GR *(24–28)* has been utilized, but low yields and instability are usually problematic. Expression of recombinant full-length receptor, in either a baculovirus expression system *(29)* or yeast *(30)*, has been reported, but only partial purification has been described, and the resulting activity has been low.

The authors' in vitro functional studies utilize highly purified GR from a one-step column purification procedure. This GR has a high affinity for its DNA element *(31)*, activates transcription *(31)*, recruits remodeling machinery

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and induces a chromatin transition on the reconstituted mouse mammary tumor virus (MMTV) promoter ***(32)***. The source of this GR is a WCL2 Chinese hamster ovary cell line that co-expresses recombinant rat GR and dihydrofolate reductase in the presence of methotrexate ***(33)***. The initial step in the purification is the production of a cytosolic extract. Nitrogen cavitation is used to lyse the cells in the presence of sodium molybdate and irreversible protease inhibitors. This provides stabilized, chaperone-associated receptor. Various extraction methods were examined; nitrogen cavitation is the only procedure that provides reproducible yields of receptor capable of binding hormone. Also, lysosomes and the nuclei remain mostly intact during this procedure, and oxidation of GR is minimized ***(34–39)***. Cytosolic extract is then incubated with the hormone, [6,7-^{3}H]-dexamethasone mesylate (^{3}H-Dex Mes), overnight at 4°C.

The next steps require optimal binding of receptor to an anion exchange column, followed by on-column transformation ***(40)***, and finally elution from the column with a salt gradient. One important aspect of this procedure is that the cytosol is loaded onto the column under high salt concentrations (260 m*M* NaCl) in the presence of molybdate, which prevents binding of many cytosolic proteins, but allows for binding of the GR with its associated proteins. By washing the column in the absence of molybdate, GR is transformed (dissociated from its chaperones) through its interactions with the anion exchange media. GR is then eluted at approx 180 m*M* salt whereas GR associated with chaperones (and the large majority of proteins still bound to the column) are eluted at approx 325 m*M*. This process provides highly pure GR from one column purification. The key to achieving large amounts of purified GR is the addition of the zwitterion detergent 3(3-cholaminopropyl diethlammonio)-1-propane sulfonate (CHAPS), which stabilizes the receptor and reduces loss by nonspecific adsorption to surfaces. A 10-fold lower concentration of CHAPS in subsequent experiments also appears to stabilize the receptor without greatly effecting the assays.

2. Materials

2.1. Cell Lysis and Column Instrumentation

1. Nitrogen cavitation bomb (Parr Cell Disruption Bomb, no. 4635, Parr Instrument, Moline, IL).
2. Compressed nitrogen tank.
3. Beckman preparative ultracentrifuge.
4. 5-cm-Diameter by 5-cm-length column packed with 100 mL Source 15Q anion exchange media (Amersham Pharmacia Biotech): As long as the back pressure is kept below 0.5 MPa, use an XK 50 column (Amersham Pharmacia-Biotech).
5. Aktadesign fast protein liquid chromatography (FPLC) system (Amersham Pharmacia-Biotech).

6. 150-mL Superloop (Amersham Pharmacia-Biotech).
7. 100 Nunc MiniSorp polypropylene low protein binding tubes (Thomas Scientific) for fraction collector.

2.2. Steroid Hormone, Cells, and Buffers

1. ^{3}H-Dex Mes (New England Nuclear).
2. WCL2 cell line: 100 L cells are grown in suspension at the National Cell Culture Center (NCCC, Minneapolis, MN); 8-L spinner flasks are cultured at 37°C in a warm room in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 1 m*M* methotrexate, and 40 μ*M* proline. Cells are harvested at ~2800*g* for 10 min, washed twice in ice-cold phosphate-buffered saline (PBS) and shipped overnight on wet ice. Cells can also be grown according to Sanchez et al. ***(31)*** in tissue culture dishes with DMEM containing 10% iron-supplemented fetal calf serum, 25 m*M* glucose, 1 m*M* methotrexate, and 350 m*M* proline at 37°C, 5% CO_2.
3. Homogenization buffer: 20 m*M* Bis-Tris, pH 7.2, 10 m*M* $NaMO_4$, 10% glycerol, 5 m*M* dithiothreitol, 1 m*M* 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF), 10 μ*M* leupeptin, 1 μ*M* pepstatin A, 10 μ*M* E-64, and 10 μ*M* bestatin.
4. Column buffer A (750 mL): 20 m*M* Bis-Tris, pH 7.2, 7.5 m*M* CHAPS, 1 m*M* AEBSF, and 2 m*M* dithiothreitol.
5. Column buffer B (750 mL): Buffer A plus 500 m*M* NaCl, and 10 m*M* $NaMoO_4$. **Note:** All water, ethanol, and buffers must be filtered through a 0.2-μ filter before running through the column or FPLC system.

3. Methods

3.1. Preparation of Cytosolic Extract

1. Upon receipt from NCCC, cells are washed twice with ice-cold phosphate-buffered saline (they are usually washed twice at the NCCC before shipping as a 100–150 mL pellet).
2. Cell pellet is resuspended in 2 cell pellet volumes of homogenization buffer.
3. Transfer the cell suspension into a Nunc 250-mL centrifuge bottle that has a hole cut in top for insertion of the cavitator outlet tube. Place the bottle in the cavitator and surround with ice. Put the cavitator in an ice bucket filled with ice.
4. Assemble the cavitator according to manufacturer's instructions (*see* www.parrinst.com/library.html#CellBombs).
5. Bring the cavitator up to 500 psi with compressed nitrogen for 15 min. Carefully collect the cell debris and cytosol into another Nunc 250-mL centrifuge bottle.
6. Centrifuge the mixture in Beckman centrifuge bottles at 100,000*g* in a Beckman 45Ti rotor at 4°C for 1 h.
7. Carefully remove the white lipid layer from the top. Collect the cytosol in 50-mL conical tubes (40 mL/tube), flash-freeze in liquid nitrogen, and store in liquid nitrogen cooled freezer (–250°C). Usually, a 100-L preparation will yield 250 mL of cytosol. Cytosol should be used within 4–6 mo of preparation.

3.2. Column Preparation

Note: If pouring a new column, follow **step 1**. If using a previously used column, proceed to **step 2**.

1. Prepare about 100 mL Source 15Q anion exchange media according to manufacturer's specifications. Pour a 5-cm-diameter by 5-cm-length column. Pack column with 1 column volume H_2O at 1 mL/min. Equilibrate the column with 5 column volume 20 m*M* Bis-Tris, pH 7.2, at 2.5 mL/min. Saturate nonspecific binding sites by loading 100 mL 2 mg/mL bovine serum albumin (BSA) (protease, nuclease-free, Calbiochem) in 20 m*M* Bis-Tris, pH 7.2, onto the column, at 2.5 mL/min. Wash with 5 column volume 20 m*M* Bis-Tris, pH 7.2, with 500 m*M* NaCl, at 5 mL/min.
2. This should be done the day before purification. To regenerate a previously used column, dissemble column and gently stir media with glass stir rod to loosen. Wash with 2 column volume 0.5 *M* ethylenediamine tetraacetic acid, pH 8.0, at 1 mL/min (this step removes the molybdate in the GR purification and homogenization buffers from the column). Wash column with 1 column volume 2 *M* NaOH, at 1 mL/min. Wash with 1 column volume 1 *M* HCl, at 1 mL/min. Wash with 5 column volume dH_2O at 5 mL/min. Dissemble column, and gently stir media with glass stir rod to loosen.
3. Repack column and regenerate anion exchange capacity with 4–5 column volume 2 *M* NaCl, at 1 mL/min.
4. Load fraction collector with 100 tubes. Set fraction collector to collect 4-mL fractions.

3.3. Hormone Binding and Purification

1. The night before purification, thaw three conical tubes (120 mL cytosol) in a 37°C water bath. Add 100 µL of ^{3}H-Dex Mes (1 mCi/mL, 49.5 Ci/mmol, 20 µ*M*) to each tube to a final concentration of 50 n*M*. Mix gently and store overnight (approx 16 h) in an ice bucket at 4°C.
2. On purification day, once the column is packed, carefully secure the top plunger to the column bed. Prepare buffers A and B and filter. Equilibrate column with 2 column volume of 60% B at 2.5 mL/min.
3. While column is equilibrating, add NaCl to the cytosol to make a final concentration of 260 m*M*. Filter the cytosol using 0.45 µ Millipore-HA low protein binding syringe filters. Multiple filters are necessary.
4. The UV detector is typically set at 280 nm.
5. Load superloop with cytosol.
6. At 2.5 mL/min, inject 20 mL cytosol, followed by washing with 10 mL 60% B. Repeat until all cytosol is loaded onto the column.
7. Wash with 1 column volume 60% B at 5 mL/min.
8. Wash column with 225 mL 0% B at 3 mL/min. This is the on-column transformation step, since molybdate is now being removed from the column-bound GR. This step should take at least 1 h.

9. Perform gradient from 0 to 100% B for 1.2 column volume at 5 mL/min. Collect 4-mL fractions.
10. Wash column with 2 column volume 100% B.
11. Collect 25-μL aliquots of each fraction and count the ^{3}H using a scintillation counter.
12. Wash superloop with filtered H_2O, then 20% ethanol. Wash pumps and column with H_2O followed by 20% ethanol. Store system in 20% ethanol.

3.4. GR Fraction Collection and Concentration

A typical UV column salt-elution profile, for the size column in this protocol, is illustrated in **Fig. 1A**. The majority of the cytosolic proteins are eliminated during loading of the column in the presence of 260 m*M* NaCl (profile not shown). The ^{3}H-Dex Mes typically elutes between fractions 28 and 36, at a NaCl concentration of about 180 m*M* NaCl (**Fig. 1B**, transformed peak). This is significantly earlier than the time the majority of the proteins are eluted. Note that a second, broader peak is also occasionally observed. This peak appears to be less pure and contains a larger portion of untransformed GR, making it less desirable for use.

1. Always passivate pipet tips by pipeting up and down repeatedly with 2 mg/mL nuclease-, protease-free BSA. Pool fractions containing peak, as described above.
2. Centrifuge in Centricon-30 concentrators that have been passivated in 2 mg/mL protease, nuclease-free BSA, 20 m*M* Bis-Tris, pH 7.2, and 7.5 m*M* CHAPS by rocking at room temperature for 1 h. Try to use as few concentrators as possible, because GR is easily lost on the membrane even after passivation.
3. Concentrate until the GR solution is at least 6000 cpm/μL. This will be about 100 n*M*, depending on the specific activity of the ^{3}H-Dex Mes. Since further concentration results in continued GR loss on the membrane, make more concentrated only if needed.
4. Calculate the GR concentration according to the following equation (first convert counts in cpm to distintegrations per minute [dpm]):

$$\frac{(\text{Counts, dpm}/\mu\text{L})(1 \times 10^6\ \mu\text{L/L})}{(2.22 \times 10^{12}\ \text{dpm/Ci})(\text{Dex Mes specific activity, Ci/mol})} = \text{GR conc. (M)}$$

5. Using BSA passivated tips, pipet GR into aliquots to prevent repeated freeze–thaws. Flash-freeze in liquid nitrogen and store in a liquid nitrogen cooled freezer.

3.5. Analysis of Purified GR

Due to the extensive on-column washing and elution prior to the main protein peak, this GR preparation is highly pure according to silver stain and ^{3}H autoradiography (**Fig. 2A,B**). However, the authors have found that some chaperone proteins remain with this GR preparation, albeit at much lower than stoichiometric amounts (unpublished results). Although near-homogeneity was

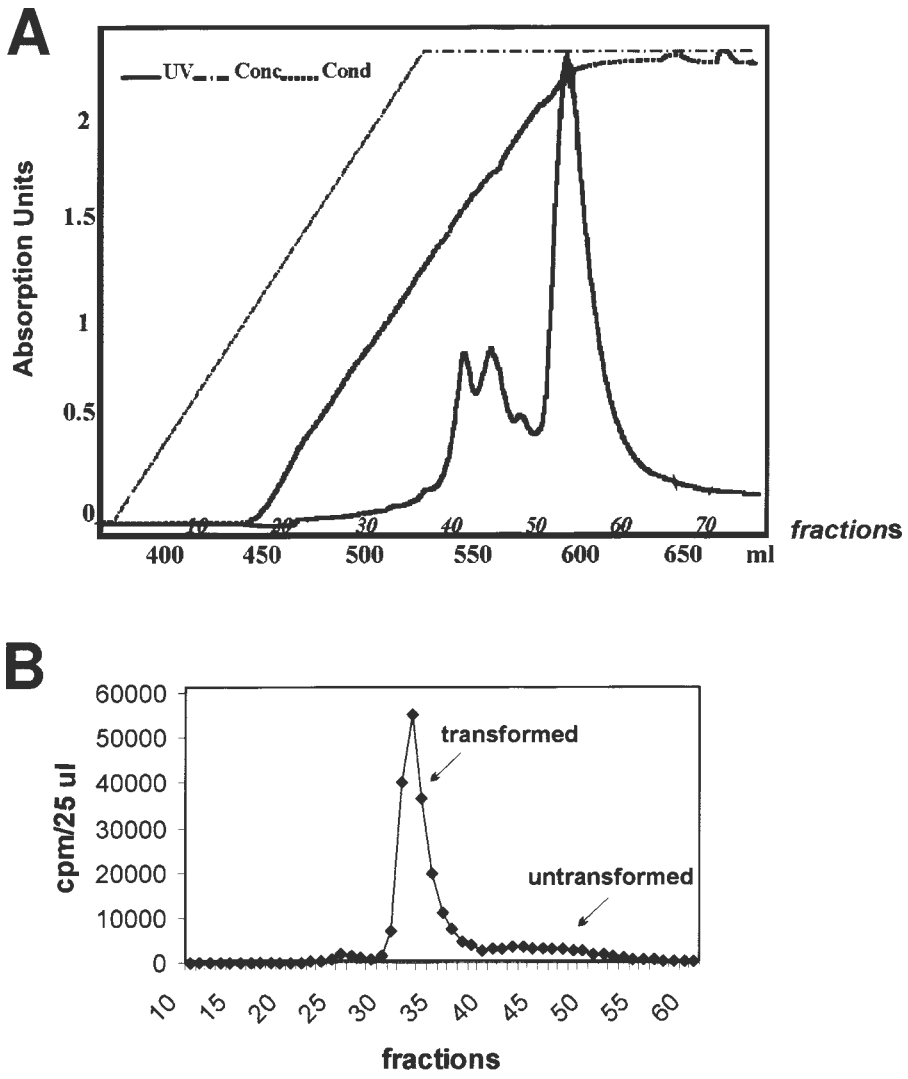

Fig. 1. Column purification of GR. **(A)** Anion exchange FPLC column profile. UV refers to the absorption at 280 nm, Conc refers to buffer concentration gradient from 0 to 100% buffer B. Cond is the conductivity in mSieverts/cm. Fraction numbers are in italics. **(B)** Detection of ^{3}H-Dex Mes-bound GR by scintillation counting. Fractions 32–34, containing chaperone dissociated GR (transformed), were pooled from this particular purification.

achieved in subsequent purification, using phenyl-sepharose chromatography, GR binding and transcriptional activity was greatly diminished ***(31)***. The

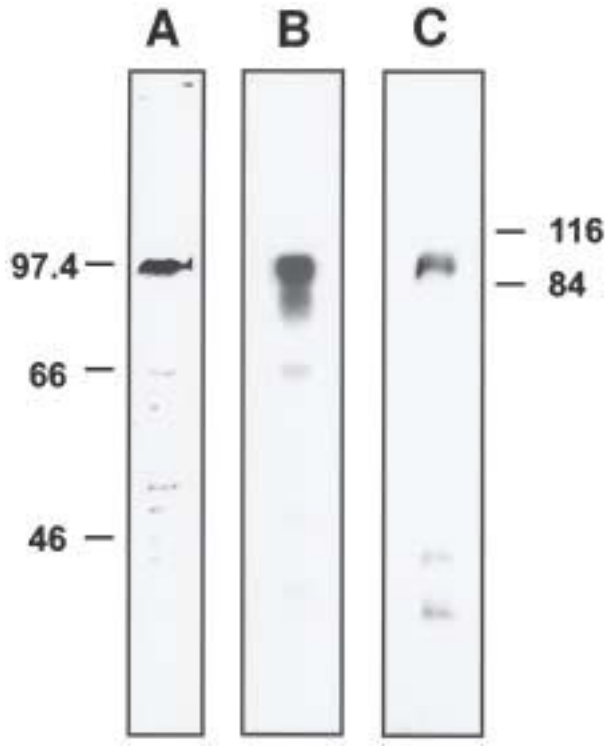

Fig. 2. Protein analysis of purified GR. **(A)** Silver stain of 8% sodium dodecyl sulfate (SDS) polyacrylamide gel. ^{3}H-autoradiography of a 8% polyacrylamide gel to detect proteins bound to ^{3}H-Dex Mes **(B)**. **(C)** Immunoblot of a 8% SDS polyacrylamide gel with PAI-512 anti-GR antibody (Affinity Bioreagents). Location of size markers are on the sides of the gels/blots.

authors analyze each GR preparation by mobility shift assay, and detect GR binding to both a glucocorticoid response element (GRE) containing double-stranded oligonucleotide (5'-CTAGGCTGTACAGGATGTTCTGCCTAG-3') and a 217-bp fragment of DNA derived from the B nucleosome region (–248 to –31) of the MMTV promoter (**Fig. 3A,B**). Finally, GR binding to magnetic bead-immobilized MMTV long terminal repeat (LTR) chromatin is detected by a block in the restriction enzyme, *Sac*I, located between GRE-2 and -3 (**Fig. 3C**) ***(32)***. Increasing GR results in a decrease in the amount of chromatin that is digested by *Sac*I (cut) compared to the undigested (uncut). The amount of GR used in subsequent experiments is determined by its performance in the mobility shift and *Sac*I block assays.

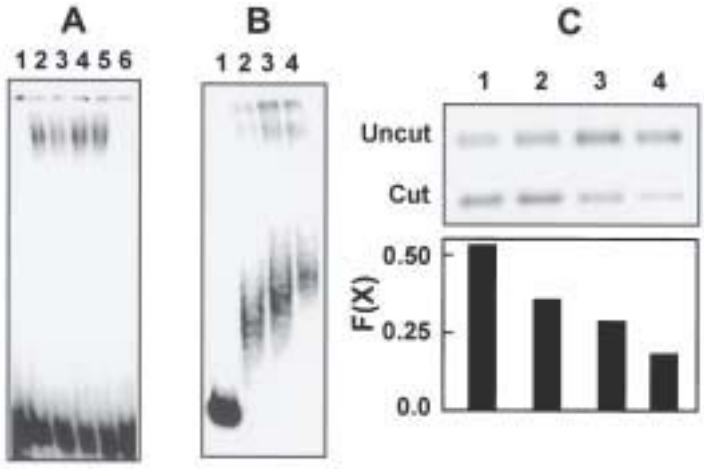

Fig. 3. GR binding to DNA and chromatin. (**A**) Mobility shift of ^{32}P-end labeled-oligonucleotide, (5'- CTAGGCTGTACAGGATGTTCTGCCTAG-3') on a 5% native polyacrylamide gel. Oligonucleotide concentration was 1.4 n*M*. *Lanes 1* and *6* are oligonucleotide alone. *Lanes 2* and *3* contain 1 n*M* GR. *Lanes 4* and *5* contain 6 n*M* GR. *Lanes 3* and *5* also have 10 n*M* cold oligonucleotide as competitor. (**B**) Mobility shift of [^{32}P]-end labeled-217-bp fragment of DNA derived from the B nucleosome region (–248 to –31) of the mouse MMTV on a 5% native polyacrylamide gel. *Lane 1* is DNA alone. *Lanes 2–4* contain 5, 15, and 50 n*M* GR, respectively. The binding reactions (20 μL) for both (A) and (B), containing 10 m*M* HEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 2 mg/mL bovine serum albumin, 10% glycerol, 1 μg poly dI·dC and 1 m*M* AEBSF, 10 μ*M* leupeptin, 1 μ*M* pepstatin A, 10 μ*M* E- 64, and 10 μ*M* bestatin, were incubated at room temperature for 20 min. Block of *Sac*I access to its site on MMTV chromatin (**C**). Chromatin was reconstituted onto magnetic bead (Dynal) immobilized MMTV promoter (1.8 kb *Nco*I/*Sph*I fragment) using *Drosophila* embryo extracts, according to Fletcher et al. ***(32)***. Chromatin was incubated with 0, 5, 15, and 50 n*M* GR (*lanes 1–4*) in the buffer conditions described above but lacking poly dI·dC at room temperature for 20 min. 10 U *Sac*I was added and the reaction was incubated at 37°C for 15 min. Reactions were deproteinated and ^{32}P-end-labeled, according to Fletcher et al. ***(32)***. The amount of *Sac*I access is measured by the fractional cleavage, F(x); the ratio of chromatin cleaved by *Sac*I (cut) divided by the total (cut + uncut).

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5

Production and Purification of Histidine-Tagged Dihydrotestosterone-Bound Full-Length Human Androgen Receptor

Mingmin Liao and Elizabeth M. Wilson

1. Introduction

Protein purification and characterization is required for a full understanding of structure–function relationships. Because proteins have complex structures and can be present at low concentrations, efficient purification protocols are needed. Purification of full-length androgen receptor (AR) is complicated by its low abundance, instability in the absence of androgen, and size and charge similarities with other nuclear proteins. Previous approaches to steroid hormone receptor purification have included traditional chromatography, such as ion exchange, gel filtration, isoelectric focusing chromatography ***(1,2)***, and hormone, DNA, and antibody affinity chromatography ***(3–5)***, but with low yield and purity. Overexpression of recombinant nuclear receptors or their domains in insect cells ***(6)***, *Escherichia coli* ***(7)***, or mammalian cells ***(8)***, has facilitated their purification. Purification with histidine (His)-tagged proteins is advantageous, because, unlike protein tags, such as glutathione *S*-transferase, short His sequences can have minimal effects on protein structure and function, efficiently bind metal-chelating columns mostly independent of protein conformation, and may not require the use of a cleavage step ***(9,10)***. This chapter details a procedure for the isolation of nondenatured, recombinant human AR with more than 95% purity using four-step chromatography with milligram yields (*see* **Notes 1–4**). Purified AR may be used in physical and biochemical studies, such as monoclonal antibody development ***(11)***, crystallography and nuclear magnetic resonance studies, DNA binding, and solution dimerization ***(6)***.

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2. Materials

2.1. Cloning and Baculovirus Expression of His-Tagged Human AR

2.1.1. Cloning

1. pAcC4 transfer vector (Cetus), store DNA at –20°C.
2. pCMVhAR vector, a mammalian expression vector: Coding for the full-length human AR ***(12)***.
3. Polymerase chain reaction (PCR) reagents and instrument.
4. Restriction enzyme and buffer, T4 DNA ligase, and buffer.
5. DH5α *E. coli* competent cells, store stocks at –80°C.
6. Luria broth (LB) medium and culture plates with 100 μg/mL ampicillin: Store at 4°C.

2.1.2. Recombinant Virus Isolation by Plaque Assay and Protein Expression

1. *Spodoptera frugiperda* Sf9 insect cell (Invitrogen, San Diego, CA): Store in liquid nitrogen.
2. Circular baculovirus, *Autographa californica* nuclear polyhedrosis virus Ac*M*NPV and transfection buffer (Invitrogen).
3. Fetal bovine serum (FBS) (Gibco-BRL): Store at –20°C.
4. Yeastolate and lactalbumin hydrolase (Difco).
5. Grace medium with additives 0.33% yeastolate and 0.33% lactalbumin hydrolysate (Lineberger Cancer Center, University of North Carolina at Chapel Hill): Grace medium stored at 4°C.
6. Plain Grace medium: Grace medium without additives.
7. Complete Grace medium: Grace medium containing 10% FBS, 70 mg/mL gentamycin, 100 U/mL penicillin, and 100 mg/mL streptomycin.
8. Incomplete Grace medium: Complete medium without FBS.
9. Pluronic F-68 (JRH Bioscience).
10. Neutral red, 10 mg/mL stock in dH_2O, filtered, and stored at 4°C.
11. SeaPlaque agarose (FMC Bioproducts), 5% stock in dH_2O, autoclaved, and stored at room temperature.

2.2. Preparation of Whole-Cell Extract

2.2.1. Stock Solutions

Stocks solutions listed (1–7) below are stored at –80°C for several months. 1 *M* Imidazole solution is stored at 4°C for 1 mo.

1. Dihydrotestosterone (DHT) (Sigma): 34.4 m*M* in ethanol.
2. Imidazole (Sigma): 1 *M* in dH_2O, pH 7.6.
3. Phenylmethylsulfonyl fluoride (PMSF) (Sigma): 0.1 *M* in ethanol.
4. Dithiothreitol (DTT) (Sigma): 1 *M* in dH_2O.
5. Leupeptin (Sigma): 5 m*M* in dH_2O.

6. Pepstatin A (Sigma): 2.8 m*M* in ethanol.
7. ε-amino-n-caproic acid (Sigma): 3 *M* in dH_2O.
8. Extraction buffer (EB) (*see* **Notes 5** and **6**): Extraction buffer (EB): 0.5 M NaCl, 10% glycerol, 0.01% NP-40, 20 m*M* Tris-HCl, pH 7.6 (all solution pH measured at room temperature). This solution can be stored at 4°C for at least 1 mo. Before use, add fresh β-mercaptoethanol to 5 m*M*, and protease inhibitors from stock solution to final concentration of 0.5 m*M* PMSF, 10 μ*M* leupeptin, 10 μ*M* pepstatin A, 20 m*M* ε-amino-n-caproic acid. Add DHT to 1 μ*M*.
9. Dialysis buffer (*see* **Notes 5** and **6**): 0.15 *M* NaCl, 10% glycerol, 0.01% NP-40, 5 m*M* imidazol, 20 m*M* Tris-HCl, pH 7.6, add 5 m*M* fresh β-mercaptoethanol, 0.5 m*M* PMSF, 20 m*M* ε-amino-n-caproic acid, 1 μ*M* DHT.

2.3. Immobilized Metal-Affinity Chromatography (IMAC)

1. Talon resin (Clontech), supplied in 1:1 (v/v) 20% ethanol.
2. Gravity column (Bio-Rad).
3. Binding buffer: EB (*see* **Subheading 2.2.2.**) with 0.15 *M* NaCl and 5 m*M* imidazole.
4. Elution buffer: EB containing 100 m*M* imidazole.

2.4. Phenyl-Sepharose Chromatography

1. Fast protein liquid chromatography (FPLC) system (Pharmacia).
2. Phenyl-Sepharose resin (Pharmacia).
3. Sample loading buffer (SLB): 0.5 *M* NaCl, 10% glycerol, 0.5 m*M* ethylenediamine tetraacetic acid (EDTA), 20 m*M* Tris-HCl, pH 7.6, add fresh 1 m*M* DTT, 1 m*M* DHT, and protease inhibitors as in EB (*see* **Subheading 2.2.2.**).
4. Washing buffer: SLB without NaCl.
5. Gradient buffer A (GB-A): SLB with 0.1 *M* NaCl.
6. Gradient buffer B (GB-B): GB-A containing 80 m*M* 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS) (Sigma).

2.5. Heparin-Sepharose Chromatography

1. Hitrap heparin-Sepharose column, 5 mL or 1 mL (Pharmacia).
2. GB-A (*see* **Subheading 2.4.**).
3. Gradient buffer C (GB-C): GB-A with 1.0 *M* NaCl.

2.6. Gel Filtration Chromatography

1. HiLoad 26/60 Superdex200pg column (Pharmacia).
2. Running buffer (*see* **Notes 6** and **7**): 0.4 *M* NaCl, 0.5 m*M* EDTA, 10% glycerol, 20 m*M* Tris-HCl, pH 7.6, add fresh 1 m*M* DTT, 1 μ*M* DHT, 0.5 m*M* PMSF.

3. Methods

3.1. Cloning and Expression of His-Tagged Human AR

3.1.1. Cloning

Full-length human AR was cloned into the polylinker region of the baculovirus transfer vector, pAcC4, with six His-tags at the NH_2-terminus ***(6)***.

1. Digest vector, pAcC4, with restriction enzymes *Nco*I and *Bam*HI.
2. Digest vector, pCMVhAR, with *Afl*II and *Bam*HI to get 2.24-kb AR C-terminal fragment.
3. PCR amplification of AR NH_2-terminal fragment with *Nco*I site and 6 His at 5' and *Afl*II site at 3'. Digest the PCR product with *Nco*I/*Afl*II.
4. Purify the three DNA fragments above and perform a triple ligation with T4 DNA ligase.
5. Transform into *E. coli* competent cells and plate on LB plate.
6. Pick colony into 5 mL LB medium and grow at 37°C overnight.
7. Make plasmid miniprep and digest with appropriate restriction enzyme, to confirm inserts.
8. Verify PCR-amplified region by sequencing analysis.
9. Make high-quality plasmid DNA (pAcC4hAR) using CsCl gradient for transfection into Sf9 cells.

3.1.2. Sf9 Cell Culture (see **Note 8**)

1. Store Sf9 cell aliquots in 10% dimethyl sulfoxide in incomplete Grace medium at 2×10^7 cells/mL in liquid nitrogen.
2. Set up cell cultures by transferring aliquots of stored cells to two T150 flasks containing 30 mL complete Grace medium. Incubate at 27°C, and change medium after 30 min–1 h, then change medium every 3 d until cells grow to 80–90% confluence (about 5 d).
3. Bang off cells from T150 flasks. Add 0.5 mL cell suspension to a T75 flask with 20 mL complete Grace medium and culture as above. Sf9 cells growing in monolayer culture can be passaged for many cycles and used as a backup for the spinner culture. Keep media stock that is used for the flasks separated from that for spinner to minimize contamination.
4. For restarting the spinner from the T75 flask, set up two T150 flasks by transferring 10 mL cell suspension from T75 flask to each T150 flask, with 35 mL complete Grace medium, and incubate at 27°C for 4–5 d (90% confluence growth).
5. Bang off cells from T150 flasks and transfer the cell suspension to 50-mL tube. Centrifuge at 3000 rpm (900*g*) 5 min.
6. Aspirate supernatant. Resuspend cell pellet in 20 mL complete Grace medium and count cell density.
7. Subculture cells in 100 mL Bellco spinner flask in complete Grace medium containing 0.1% Pluronic F-68 with starting density of $0.5–0.7 \times 10^6$ cells/mL using a Bellco magnetic stirrer. Incubate at 27°C for 3 d with robust stirring (about 2 rps). Passage cells every 3 d as above. Cells at d 3 are at log growth phase and used for virus infection.

3.1.3. Transfection and Purification of Recombinant Virus by Plaque Assay

1. Dilute cells from the spinner at d 3 with incomplete Grace medium to 0.7×10^6 cells/mL. Plate 3 mL (2×10^6 cells) in 60 × 15 mm cell culture dishes. Put plates

at 27°C for 30 min to allow cells to settle. Set up two control plates for the wild-type (WT) Ac*M*NPV virus only or without any viral DNA. During the incubation, make transfection mixture: 1 μg circular Ac*M*NPV wild type viral DNA, 5 μg pAcC4hAR DNA, and 0.75 mL transfection buffer (1X in plain Grace medium with 10% FBS).

2. After cells settle, aspirate medium to dryness. Gently add the transfection mixture to the center of plate. Rock plates at slow speed on platform rocker 1 h at room temperature.
3. Prepare 0.7% SeaPlaque agarose solution and carefully add 5–6 mL/6 cm plate. Incubate plates at 27°C for 5–6 d. (20 mL 0.7% agarose solution: Prewarm 17.2 mL complete Grace medium in 50°C water bath, heat in microwave to dissolve 5% SeaPlaque agarose stock, and put 2.8 mL into prewarmed medium.)
4. At d 5 or 6, put 2 mL neutral red agarose overlay into the center of the plate. Make sure the agar is spread evenly. Incubate plates at 27°C overnight (10 mL overlay: 9 mL complete Grace medium, 1 mL 5% SeaPlaque agarose stock, 0.2 mL 10 mg/mL neutral red stock).
5. Check plates under the microscope for recombinant plaques by comparing with the plaque appearance in plate with WT virus, which causes expression of opaque occlusion bodies that can be readily apparent in the microscope.
6. Under sterile conditions, pick five recombinant plaques using a 1-mL Pipetman tip pushed vertically to the bottom of the plate, and carefully aspirate the agar plug. Place the plug in 1 mL plain Grace medium and incubate at room temperature 20 min and vortex to release virus from the gel. Set up serial dilution in incomplete Grace medium 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} for next round of plating because plaques may contain WT virus contamination. Plates can be stored temporarily at 4°C by sealing with parafilm.
7. Repeat plaque assay by infecting plated cells with 0.6 mL virus from previous step. Another 1–2 rounds may be needed for plaque isolation. If plaques in a plate are well-separated and all are recombinant, the picked plaque from the plate is considered a pure recombinant virus. Otherwise, additional rounds of plaque purification should be done. Pick five purified plaques for baculovirus grow-up.

3.1.4. Recombinant Baculovirus Grow-Up

1. Plate Sf9 cells from the spinner culture in 24-well tissue culture plates at a density of 10^5 cells/well with incomplete medium and incubate at room temperature for 20 min.
2. Aspirate medium. Carefully add 0.8 mL complete medium/well and a pure virus plug from **Subheading 3.1.3., step 7**.
3. Seal the plates with parafilm and incubate at 27°C for 5 d.
4. At d 5, harvest virus by transferring the medium to 1.5-mL microcentrifuge tubes and microcentrifuge 1 min. Collect the supernatant in an Eppendorf tube and store in the dark at 4°C as the first viral stock.
5. Check protein expression as in **Subheading 3.1.5.** by immunoblot. Plate 3.5×10^6 cells in 6-cm dish as above and infect cells with 100 μL virus from the first viral stock.

6. Make second viral stock in T25 flasks. Plate 4×10^6 cells per T25 flask in 4 mL incomplete Grace medium and allow cells to settle. Aspirate medium and add 1 mL incomplete medium and 100 μL virus from the first viral stock. Rock 1 h at room temperature. Add 4 mL complete Grace medium and incubate at 27°C for 5 d.
7. Bang off cells and transfer to 15-mL tube. Centrifuge 1000*g* 5 min and collect supernatant. Store at 4°C as second viral stock.
8. Further expand the virus in T75 flasks with 9×10^6 cells and 15 mL complete medium as third viral stock and for protein expression.

3.1.5. AR Expression by Adherent Cell Culture (see **Notes 9–11**)

1. Determine cell density of the suspension culture at d 3 and use 2.6×10^7 cells/15-cm dish.
2. Add incomplete Grace medium to total volume per dish of 15 mL.
3. Add cell suspension and incubate the plate at 27°C for 20 min to allow cells to settle.
4. Aspirate medium and add 3 mL incomplete Grace medium, being careful not to disturb the cell layer. Add recombinant baculovirus at a multiplicity of infection (MOI) of 1–5. Incubate plates on platform rocker for 2 h at room temperature for even infection.
5. Carefully add 15 mL complete Grace medium and incubate plates at 27°C overnight.
6. Add DHT to a final concentration of 1 μ*M* and continue incubation for 48 h.

3.2. Preparation of Whole-Cell Extract

3.2.1. Harvest Cells from Adherent Culture

1. Aspirate medium and wash cell layer with 7 mL ice cold phosphate-buffered saline. Aspirate the solution and add 1 mL ice-cold phosphate-buffered saline, and scrape cells into solution using rubber policeman.
2. Transfer cell suspension into 1.5-mL microcentrifuge tubes and centrifuge at 13,000 rpm (16,060*g*) at 4°C for 3 min. Cell pellets can be frozen at –80°C for several months or proceed to whole-cell extract.

3.2.2. Whole-Cell Extracts

1. Resuspend cell pellets in 1 mL EB 15-cm plate.
2. Freeze and thaw three times and incubate on ice for 1 h.
3. Transfer the cell lysis into precooled Beckman centrifuge tubes. Centrifuge at 143,000*g* for 45 min. Collect supernatant for chromatography (supernatant can be frozen at –80°C for weeks without obvious degradation of AR).

3.3. Immobilized Metal-Affinity Chromatography (see **Notes 12 and 13**)

1. Start with 50 mL cell extract (50 15-cm plates). Dialyze the cell extract against dialysis buffer in a volume ratio of 1:20 for 2 h at 4°C. Centrifuge to clarify supernatant as above.

2. During the dialysis, prepare the Talon resin, which is supplied in 1:1 vol 20% ethanol. Place 4 mL of the mixture into a 50-mL Falcon tube and spin at 700*g* 5 min. Carefully pour off ethanol. Resuspend beads in 20 mL binding buffer and spin again. Equilibrate resin with 20 mL binding buffer for 30 min with gentle agitating. Spin and pour off buffer. The resin is ready for use.
3. Put 25 mL cell extract into 2 mL preequilibrated Talon resin and incubate 45 min with gentle agitation. Transfer the mixture to a 20-mL gravity column. Adjust the outflow rate to 1.5 mL/min. Decant the solution, making sure the surface of the bed is not dry.
4. Wash resin with 20 mL binding buffer and carefully apply buffer without disturbing the bed surface.
5. Elute bound protein with 40 mL elution buffer, which will collect most bound protein.
6. Make aliquots from each fraction for analysis such as protein staining, immunoblot analysis, protein quantitation, AR enzyme-linked immunosorbant assay (ELISA), and DNA binding.

3.4. Phenyl-Sepharose Chromatography (see Notes 12, 14, and 15)

1. Pack a 10-mL column or use prepacked column. Capacity of 10 mL resin is sufficient for binding AR from 50 15-cm plates.
2. Set FPLC flow rate at 1.5 mL/min. Equilibrate column with 100 mL SLB.
3. Load sample from Talon elution, about 80 mL.
4. Wash with SLB until OD_{280} reaches baseline, usually 50 mL.
5. Wash with washing buffer to OD_{280} baseline, usually 50–70 mL.
6. Reequilibrate the column with 50 mL GB-A.
7. Further wash the column with 4 m*M* CHAPS solution by programming to mix 95% GB-A and 5% GB-B for about 50 mL. This fraction may contain loosely associated AR.
8. Elute bound AR with 25 m*M* CHAPS solution by programming to mix 69% GB-A and 31% GB-B, until OD_{280} approaches the baseline. A typical profile is shown in **Fig. 1A**.

3.5. Heparin-Sepharose Chromatography (see Notes 12, 14, and 16)

1. Set FPLC flow rate at 1 mL/min. Equilibrate heparin-Sepharose column with 50 mL GB-A.
2. Load AR eluent from phenyl-Sepharose.
3. Wash column with GB-A until OD_{280} to baseline, about 20 mL.
4. Elute bound AR with 0.3 *M* NaCl solution by programming to mix GB-A and GB-C. Most bound AR will be recovered in a <10 mL fraction (profile in **Fig. 1B**).

3.6. Gel Filtration Chromatography (see Notes 14 and 17)

1. Calibrate new column with protein markers, to define void volume and Stokes radius.
2. Set FPLC flow rate at 1 mL/min. Equilibrate column with 500 mL running buffer.

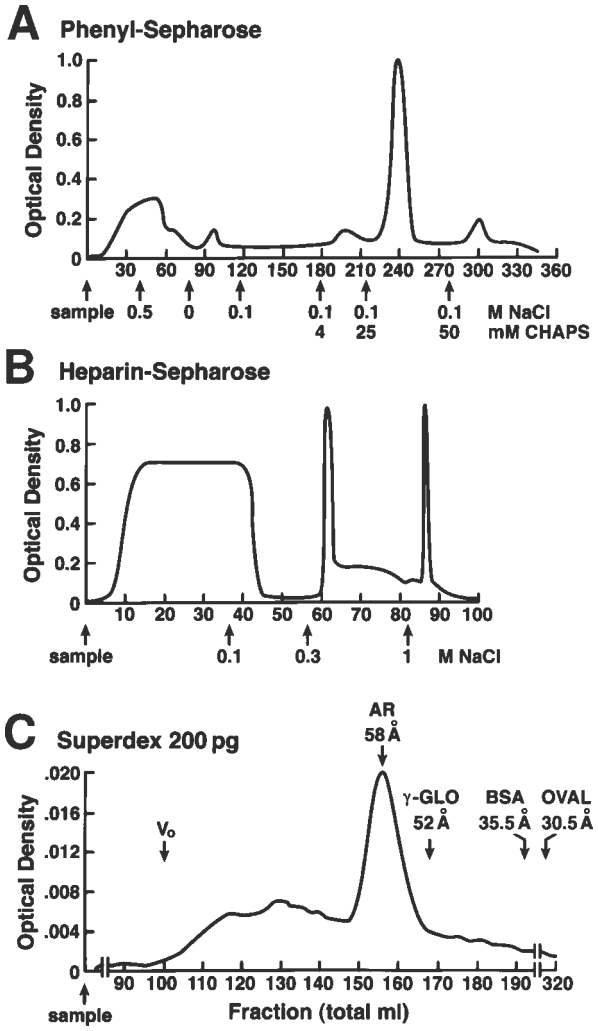

Fig. 1. Chromatographic profiles of human AR purification from Sf9 cells. Typical chromatographic profiles are shown following chromatography of the metal affinity elution sample onto **(A)** phenyl-Sepharose, **(B)** heparin-Sepharose, and **(C)** Superdex 200 pg. Elution patterns are shown relative to the total volume in milliliters (*x*-axis). The position of buffer changes (panels A and B) and approximate Stokes radius, relative to the void volume V_0 (panel C), are indicated. AR was detected by immunoblot, and quantitated by ELISA of peak fractions from phenyl-Sepharose (200 mL), heparin-Sepharose (67 mL), and Superdex 200 pg (156 mL). Optical density was determined at 280 nm. Reprinted with permission from **ref. *11***, Copyright 1999, American Chemical Society.

3. Load AR elution from previous step onto the column, and elute protein in running buffer. AR elutes as a peak of 150–165 mL with Stokes radius 58 Å (profile in **Fig. 1C**).

3.7. Column Concentration of Purified Recombinant Human AR (see Note 7)

1. A small heparin-Sepharose column (1 mL) (*see* **Subheading 2.5.**) is used for concentration purified AR.
2. Dialyze purified AR at 4°C in 2 L GB-A (*see* **Subheading 2.4.**).
3. Set FPLC flow rate at 0.5 mL/min. Equilibrate column with 10 mL GB-A.
4. Load AR sample and wash column with 2 mL GB-A.
5. Elute AR with GB-A, but 0.5 *M* NaCl, AR peak within 2 mL with >90% recovery.

4. Notes

4.1. General Considerations

1. Time consideration: The procedure requires 4 d starting from harvesting cells, but ideally column purification would be continued through the night to minimize AR degradation. If necessary, protein samples can be stored frozen –80°C between steps and thawed rapidly before use.
   a. D 1, protein extraction.
   b. D 2, dialysis and IMAC.
   c. D 3, phenyl-Sepharose.
   d. D 4, heparin-Sepharose and gel filtration chromatography.
2. Typical results with 50 15-cm plate starting material is shown in **Table 1** and **Fig. 2**, and typical FPLC profile shown in **Fig. 1**.
3. This protocol is suitable only for the androgen-bound AR purification, because the ligand-free AR undergoes extensive degradation during the purification procedure.
4. Ion-exchange, such as Mono-Q or Mono-S, and isoelectric focusing chromatography were not useful for separating Sf9-expressed AR from other nuclear protein.
5. AR is susceptible to oxidation. Reducing agents should be kept active in all solutions by adding fresh and keeping at 4°C.
6. AR is subject to protease degradation. Precool all buffers and handle protein samples at 4°C. Protease inhibitor cocktails should be added, particularly in crude extracts and the IMAC step. After the heparin-Sepharose chromatography step, protease inhibitors can be reduced to minimize cost. Leupeptin and pepstatin A were omitted in the gel filtration chromatography and subsequent steps to minimize peptide contamination.
7. Purified AR tends to aggregate in solution. Containers for purified AR should be siliconized, and solutions containing salt concentration of 50–500 m*M* will minimize aggregation. Small-column chromatography with step elution described in **Subheading 3.7.** was most successful for concentrating the AR.

Table 1
Purification of Histidine-Tagged Baculovirus Expressed Human AR from Sf9 Insect Cells

Column	[Protein] μg/mL	Total protein (mg)	[AR] μg/mL	Total AR (mg)	Purity %	Fold	Recovery %
Cytosol	53180	5318	105	10.5	0.2	1	100
IMAC	369	14.7	195	7.8	53.1	266	74
Phenyl	188	7.5	117	4.6	61.2	306	44
Heparin	138	2.5	120	2.2	88.7	444	21
Superdex	30	0.7	—	0.7	95.0	475	6.5

Protein concentration was determined by Lowry and Bradford methods with bovine serum albumin as standard. AR was quantitated with ELISA with AR-M1 monoclonal antibody ***(11)***. A final AR purity of 95% was estimated on the basis of the apparent single band on Coomassie blue staining and a standard curve with AR from the final purification step. Optical absorption at 495 nm in ELISA was compared to the standard curve. Reprinted with permission from **ref. *11***, Copyright 1999, American Chemical Society.

4.2. Protein Expression

8. Check and record cell density and viability of the spinner culture at each passage. If the cell count drops significantly, or the cells begin to aggregate, start a new spinner culture from a frozen aliquot or from cells cultured as a monolayer. The spinner should be routinely restarted after several months.
9. Protein expression levels are critical for the success of purification. High quality cells and the appropriate amount of virus are important factors. Cells must be in log-phase growth, usually in d 3 in suspension culture for Sf9 cells. It is necessary to titrate recombinant virus using the plaque assay and adherent culture protocols to optimize MOI using immunoblot analysis.
10. Optimal large scale AR expression can be obtained using a 5-L bioreactor spinner culture. The MOI is similar to that described here and procedures for isolation are the same, except on a larger scale.
11. AR expression levels should be verified before initiating purification, especially in large-scale production. With 40 μL from whole cell extracts, perform immunoblot analysis comparing with a high AR expression control.

4.3. Chromatography

12. Chromatography profile for each step (IMAC, phenyl-Sepharose, and heparin-Sepharose) should be optimized on a small scale.
13. IMAC: Check the list of resin chemical compatibility. Do not use DTT or EDTA in solutions. Regenerated Talon resin is not encouraged, although it binds His-tagged AR well. The resulting elution contains more contaminated proteins. AR in the flowthrough does not bind resin well when reapplied to new beads. Phenyl-Sepharose, heparin-Sepharose, and gel filtration columns can be reused. Immediately after use, columns are cleaned and stored in 20% ethanol.

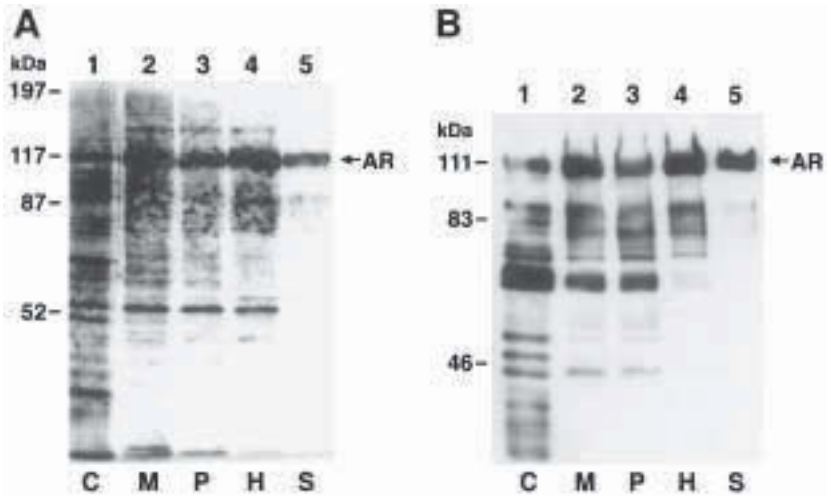

Fig. 2. Purification of baculovirus-expressed AR. AR was purified as the procedures described and as shown in **Fig. 1**. (**A**) Silver staining of the following column peak fractions: Sf9 cell cytosol (*lane 1*, C, 10 μg protein) expressing the His-tagged, human AR was chromatographed on a cobalt metal affinity Talon column (Clontech), and peak fractions eluted in IMAC elution buffer were analyzed (*lane 2*, M, 5.5 μg protein). The metal column peak fractions were applied to phenyl-Sepharose, and the AR-containing fraction at 240 mL is shown (*lane 3*, P, 3.5 μg protein). Phenyl-Sepharose AR fractions were chromatographed on heparin-Sepharose and column fraction at 67 mL containing AR is shown (*lane 4*, H, 3.5 μg protein). Heparin-Sepharose fractions were separated by Superdex 200 pg gel-filtration chromatography and the column fraction at 156 mL elution volume is shown (*lane 5*, S, 1 μg protein). Silver staining was performed with Silver Stain Plus from Bio-Rad. **(B)** Immunoblot of column peak fractions, including cytosol (*lane 1*, C, 10 μg protein); metal affinity chromatography (*lane 2*, M, 5.5 μg protein); phenyl-Sepharose peak fraction 240 (*lane 3*, P, 3.5 μg protein); heparin-Sepharose peak fraction 67 (*lane 4*, H, 3.5 μg protein); Superdex 200 pg peak fraction 156 (*lane 5*, S, 1.0 μg protein). The immunoblot was analyzed with 1 μg/mL, AR52 antipeptide antibody. Reprinted with permission from **ref. *11***, Copyright 1999, American Chemical Society.

14. Manipulation of FPLC is discussed in the user manual. All solutions for FPLC are filtered. Degassing solutions is not necessary, but temperature must be equilibrated to the same as the column and pump to prevent bubble formation in the column and tube system.
15. Phenyl-Sepharose: His-human AR binds tightly to phenyl-Sepharose beads. Bound AR is not eluted by low salt or ethylene glycerol, but by CHAPS. The authors took this advantage to extensively wash the column to eliminate contaminated proteins. Caution should be taken in 4 m*M* CHAPS washing because AR binds less tightly to the resin, which, if not regenerated well, the AR

sample may be lost. Always collect this fraction. If it contains sufficient AR, dialyze against loading buffer (*see* **Subheading 2.4.**) and rerun this step with a newly packed column. Do not shake solutions containing CHAPS to avoid excessive bubble formation.

16. Heparin-Sepharose: Step-elution is preferred to minimize the elution volume. However, gradient elution will recover more AR. If the volume of the AR eluent is too large for the subsequent step (*see* **Note 17**), a concentration step may be required (*see* **Subheading 3.7.**).
17. Gel filtration: The resin volume of the column used here is 320 mL with void volume 100 mL. Make sure the sample volume does not exceed 4% of the column volume. Flow rate is also important for optimal separation. A flow rate that is too fast decreases resolution; if too slow it increases the chance for protein degradation.

Other related protocols not described in this chapter include immunoblot, protein quantitation, SDS gel electrophoresis, protein silver staining, ELISA, and DNA-binding gel shift assay.

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6

Large Scale Production of Nuclear Receptor Ligand-Binding Domains

Li-Zhi Mi and Fraydoon Rastinejad

1. Introduction

The nuclear receptor superfamily is composed of over 150 inducible transcription factors, most of which ***(1–2)*** do not have well characterized ligands to date. Nuclear receptors regulate promoters through specific protein–protein and protein–DNA interaction at their hormone response elements, and up- or downregulate their target genes in a ligand-dependent manner with the aid of various cofactors ***(2–4)***. The high-affinity binding of ligands sets into action the complex signal transduction properties of these proteins. Ligand occupancy is a key determinant of function, because it can result in the formation of distinct molecular surfaces that have enhanced affinity for coactivators or corepressors ***(5–7)***. However, additional levels of regulation can be achieved through interactions with other systems, such as molecular chaperones, cyclic adenosine monophosphate-regulated kinases and the AP-1 activator ***(8–9)***. All of these interactions together form a rich and elaborate network by which the ligands ultimately exert their powerful effects on gene expression.

The receptor polypeptides share a common modular organization with domains A–F ***(1,2,10)***. Because the vast majority of nuclear receptors have, as yet, no characterized ligands, there is a need to understand the activation signals for so-called "orphan receptors" using biochemical and molecular tools. Various activities associated with the effects of ligand can be understood using domain E alone, which harbors the entire ligand-binding function ***(11–14)***. Therefore, to better elucidate the molecular properties associated with ligand-binding, one first needs to overexpress and purify large quantities of such proteins. This chapter explains the tools and methods that allow the production

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and rapid isolation of large quantities (milligrams) of receptor ligand-binding domains (LBDs).

The nuclear receptor LBDs are typically 20–25 kDa in size, with a common three-dimensional fold in their backbone structures and distinct recognition elements for binding their specific ligands ***(15)***. A number of crystal structures have illustrated that a common all-α-helical fold is shared by RXR, RAR, TR, RAR, ER, PR, and PPAR LBDs ***(5,6,16–20)***. The F-domain, immediately C-terminal to the LBD, encodes a structure that can switch conformation via a mouse-trap mechanism that helps further engage the ligand bound in the central core, giving rise to the so called activation function-2 in many receptors ***(17)***.

Biochemical and genetic studies have suggested that agonists and anatagonists can induce distinct conformational changes within regions E/F of the receptors ***(6,21–23)***. The magnitude of these conformational changes reflects the large size and distinct molecular properties of steroids and other hormones. Unlike the water-soluble peptide hormone and growth factors, which bind exposed regions of cell surface receptors, the ligands of nuclear receptors are lipophilic. This property gives the ligands the necessary ability to pass through the lipid bilayer of the cell membrane and reach the cognate receptors in the cell. The limited solubility of the ligands in vivo is overcome by using specific carrier proteins, such as the cellular retinoic acid-binding proteins. The ligand binding to the receptor also requires a distinctly hydrophobic binding site within the receptor polypeptides. Structural analysis of steroid receptor LBD complexes with their ligands suggests that the major forces stabilizing the protein–steroid complex are hydrophobic and van der Waals interactions ***(5,11,17–20)***.

This chapter describes a useful approach for overexpression and purification of the steroid receptor and nuclear receptor LBDs. Because bacterial expression systems typically produce the highest yields of homogenous protein, focus here is on these systems. In the authors' laboratory, expressions of LBDs of VDR, RXR, RAR, LXR, HNF-4, and AR, using bacterial expression systems, have consistently led to high protein yields (>1 mg protein/L of culture). In most cases, the purification is considerably simplified when one uses expression systems that produce in-frame fusion of the LBDs with hexa-histidine (His) or other tags useful for one-step-affinity purification (*see* **Subheading 2.1.**).

By contrast, the greater difficulty has always been in obtaining LBD polypeptides in soluble form. The hydrophobic character and large size of the steroid molecules immediately suggests the apo-receptors would have a substantial and exposed hydrophobic region reducing the solubility of the LBD. This problem is circumvented in vivo because many receptors form stabilizing

interactions with heat-shock proteins in the cytoplasm when the ligands is unavailable. The problem can be further minimized during protein isolation, in some cases, by including adequate concentrations of the ligand in the purification buffers. Nevertheless, some receptor LBDs accumulate mainly in the insoluble fraction of *Escherichia coli* lysates. For this reason, the authors also describe the methods necessary to isolate and refold LBDs that appear in the insoluble fraction of cell lysates.

2. Materials

2.1. Choosing an Expression System

The expression vector should allow large-scale protein production (with the help of a strong promoter), and simplify the subsequent purification of proteins, if possible. With purification needs in mind, there are a number of commercial vectors that are useful for producing proteins with N- or C-terminal hexa-His tags, or tags with glutathione-*S*-transferase or chitin. The authors have relied on a number of pET vectors (Novagen) that use inducible T7 promoters to drive the expression of His-tagged fusion proteins (such as pET-15b, pET-16b, and pET-21d).

The affinity purification of His-tagged proteins, using nickel (Ni)- containing columns (such as Ni-NTA His-binding resin from Novagen), is efficient whether the expressed protein requires isolation under native conditions (if the LBD is soluble) or denaturing conditions (if the protein is expressed in inclusion bodies or forms large aggregates). This is an important consideration, because the need to rely on denaturing conditions, and to subsequently refold the protein is often the case for the steroid receptor LBDs, which often appear in the insoluble portion of the cell lysates. Another advantage afforded by the His-tag constructs is the small, unobtrusive size of the tag itself, which can be left intact without compromising the function of the LBD. The ability to avoid a protease (such as thrombin or factor Xa) to excise a bulky fusion protein also reduces the risk of secondary proteolysis within the LBD sequence.

2.2. Purification of LBDs Under Nondenaturing Conditions

1. Lysis buffer: 50 m*M* phosphate buffer, pH 7.4, 300 m*M* NaCl, 10 m*M* imidazole, 0.5% Triton X-100, 1 m*M* 3(3-cholaminopropyl diethylammonio)-1-propane sulfonate (CHAPS), 5 m*M* β-mercaptoethanol (βME), 5% glycerol, with freshly added phenylmethylsulfonyl fluoride (PMSF) and benzimidine-HCl, to final concentrations of 3 and 2 m*M*, respectively (*see* **Note 1**).
2. Column wash 1: 50 m*M* phosphate buffer, pH 7.4, 300 m*M* NaCl, 50 m*M* imidazole, 1 m*M* CHAPS, 1% glycerol, 5 m*M* βME (*see* **Note 2**).
3. Column wash 2: Same as above, but containing 75 m*M* imidazole.

4. Elution buffer: Same as above, but containing 400 m*M* imidazole.
5. Final (G-25) buffer: 25 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-etharesulfonic acid, pH 7.4 (other buffers can be substituted), 100 m*M* NaCl, 1 m*M* CHAPS, 1% glycerol, 5 m*M* βME, 5 m*M* EDTA (*see* **Note 3**).

2.3. Purification of LBDs Under Denaturing Conditions

1. Denaturing lysis buffer: 100 m*M* phosphate buffer, pH 8.0, 8 *M* urea, 10 m*M* imidazole, 300 m*M* NaCl, with freshly added PMSF and benzamidine-HCl to final concentrations of 3 m*M* and 2 m*M*, respectively.
2. Denaturing column wash: 100 m*M* phosphate buffer, pH 7.4, 8 *M* urea, 10 m*M* imidazole (*see* **Note 4**).
3. Refolding buffer A: 100 m*M* phosphate buffer, pH 7.4, 4 *M* urea, 10 m*M* imidazole (*see* **Note 5**).
4. Refolding buffer B: 20 m*M* Tris-HCl, pH 7.4, 500 m*M* NaCl, 20% glycerol.
5. Ni-column wash A: 50 m*M* phosphate buffer, pH 7.4, 60 m*M* imidazole, 300 m*M* NaCl.
6. Ni-column wash B: 50 m*M* phosphate buffer, pH 7.4, 75 m*M* imidazole, 300 m*M* NaCl.
7. Elution buffer: 50 m*M* phosphate buffer, pH 7.4, 400 m*M* imidazole, 300 m*M* NaCl.
8. Final (G-25) buffer (same as above).

3. Methods

3.1. Designing the Optimal Boundaries for LBDs

There are two useful approaches in designing the optimal boundaries for a receptor LBD. First, one should consult a sequence alignment of the receptor LBDs that takes into account all of the necessary structural elements required to produce a stable protein-fold with the ligand-binding determinants ***(15)***. In some cases, the sequence comparison reveals unique insertion sequences or loops that may have special functional implications. If the activation function-2 region (domain F) is required for subsequent biochemical studies, one should consider constructs that extend farther toward the C-terminus of the receptor.

A second approach to delineating the optimal boundaries relies on biochemical identification of a limit-digest resulting from the proteolysis of the intact receptor in the presence of bound agonists or antagonists. A number of studies have shown that these limit digests occur only in the presence of bound ligand ***(22)***. Furthermore, distinct fragments are likely to result when the receptor is bound to agonists and antagonist, if these ligands protect different regions of the LBD against proteolysis. When the protease-resistant fragment is analyzed by N-terminal sequencing (8–10 residues is sufficient) and mass spectroscopy, the limits of the domain necessary for ligand binding can be defined.

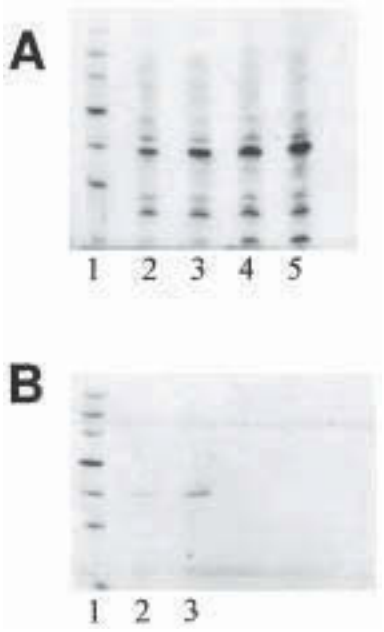

Fig. 1. Expression pattern and purification of hLXRβ LBD. **(A)** Effect of induction time on protein yield. *Lane 1* shows the standard molecular weight marker. *Lane 2* shows protein expression prior to adding IPTG. *Lanes 3–5* show expression of the protein after 1, 2, and 5 h of induction by IPTG. The major band in the gel is the hLXRβ LBD and purifies on a Ni-NTA column. **(B)** Purification of LXRβ LBD from the insoluble fraction of *E. coli* using method described in **Subheading 3.4.**

3.2. Bacterial Expression of Ligand-Binding Domains

Figure 1A shows the accumulation of a nuclear receptor LBD as a function of induction time. The following technique was applied.

1. Transform the expression vector into host BL21-DE3 cells (Novagen). Electroporation is a highly efficient and reliable technique for this purpose (*see* **Note 6**).
2. Inoculate 50 mL culture of Luria broth media, supplemented with 100 μg/mL ampicillin (substitute the antibiotic, if appropriate). Grow overnight at 37°C until saturated.
3. Next morning, inoculate 2 L of ampicillin containing Luria broth media with 50 mL overnight culture. Continue to grow at 37°C to an optical density (OD_{600}) of 0.6. (To ensure good aeration, use 6-L flasks filled with 2 L Luria broth, with vigorous shaking.)
4. When OD_{600} of the culture has been reached, induce with isopropyl thioglactose (IPTG) at a final concentration of 0.5 m*M*. Because the solubility of the receptor

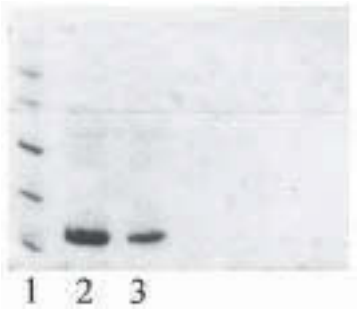

Fig. 2. Purification of LXRα LBD, from the soluble fraction of *E. coli* lysates, using the method described in **Subheading 3.3.** *Lane 1* is molecular weight markers, *lanes 2* and *3* are two peak fractions eluted from the Ni-NTA column.

LBDs can often be improved by inducing at reduced temperature, you may wish to continue this phase at 25°C (*see* **Note 7**).
5. Grow the culture for 4–5 h at 25°C (*see* **Note 8**).
6. Centrifuge culture (at 5000*g*, 25 min, 5°C) to pellet cells.
7. Scrape the cells from the bottom of the centrifuge tubes with a spatula, and drop place into a container with liquid nitrogen for rapid freezing.
8. Discard the liquid nitrogen and place the pellet in a storage container at –20°C until ready to purify.

3.3. Purification of LBD from Soluble Fraction

Figure 2 shows an example of a receptor LBD purified using the following technique:

1. Resuspend the frozen cell pellet in native lysis buffer, and allow it to thaw in a 5°C environment. The authors recommend using ~10 mL lysis buffer for each gram of frozen cell pellet, but will use more even more buffer (up to 100 mL/g) for cases in which this improves the solubility of the LBD.
2. Sonicate (or otherwise disrupt) the thawed cells on ice. Use a number of sonication periods with intermittent waiting periods to prevent warming of samples. Continue until the cell suspension is again smooth and the cells are completely lysed.
3. Transfer the material to centrifuge tubes and spin at 25,000*g* for 60 min, collect the supernatant.
4. While waiting for the centrifugation, remove a small amount (2–5 mL) of Ni-binding resin (Novagen or Qiagen) to a tube in a 5°C environment. Each 1 mL of Ni-NTA resin typically binds 4–10 mg His-tagged protein.
5. Equilibrate/wash the Ni-NTA resin by repeated suspension in 10–20 mL quantities of lysis buffer and gentle centrifugation, to settle out the resin.
6. Add the cell supernatant to the pre-equilibrated Ni-NTA resin and gently mix for 1–2 h at 5°C.

7. Load the mixture into a small column to settle the resin and allow the lysate to flow away.
8. Wash column with 20X column volume lysis buffer.
9. Wash resin with 20X column volume column wash 1.
10. Wash resin with 20X column volume column wash 2.
11. Elute the protein in 1-mL fractions using elution buffer.
12. Measure OD_{280} of these fractions to identify protein-containing samples.
13. Identify the LBD-containing fractions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of samples with significant OD_{280}.
14. Equilibrate a 5 mL Sephadex G-25 column with 5 column volume final buffer.
15. Load the peak (1 mL) fraction from Ni-NTA column onto G-25 column.
16. Elute G-25 column with final buffer and collect 2-mL fractions This step exchange the solution for storage purposes. Store peak fractions at 5°C.
17. Run SDS-PAGE to determine protein integrity prior to use (*see* **Note 9**).

3.4. Purification of LBD from Insoluble Fraction Using Denaturing/Refolding Technique

Figure 1B shows the recovery of a nuclear receptor LBD derived from the insoluble portion of *E. coli* lysate. The following procedure was used.

1. Follow **steps 1–3** in **Subheading 3.3.** in preparing the sample, but keep the pellet after centrifugation. Then add 20–30 mL denaturing lysis buffer to the pellet and sonicate until the material is well suspended. Centrifuge as before and keep the supernatant.
2. Equilibrate/wash Ni-NTA as described above, but using denaturing lysis buffer.
3. Add supernatant to preequilibrated Ni-NTA resin and mix by inversion for 1–2 h at 5°C.
4. Pour the slurry into a column, allowing the resin to pack and the solution to flow away.
5. Wash resin with 20X column volume denaturing lysis buffer.
6. Wash resin with 20X column volume denaturing column wash.
7. Begin refolding the protein by flowing refolding buffer A through the column.
8. Wash resin with 20X column volume refolding buffer B.
9. Wash resin with 20X column volume Ni-column wash A.
10. Wash resin with 20X column volume Ni-column wash B.
11. Elute the protein in 1 mL fractions using elution buffer.
12. Identify the LBD-containing fractions using SDS-PAGE analysis of samples with significant OD_{280}.
13. Equilibrate a 5-mL Sephadex G-25 column with final buffer.
14. Load the protein peak (1 mL) from Ni-NTA column onto the G-25 column.
15. Elute G-25 column in 2-mL fractions with final buffer and store peak fractions at 5°C.
16. Run SDS-PAGE before use to analyze integrity of protein.

4. Notes

1. CHAPS, Triton TX-100 and other detergents, as well as glycerol, can improve the yield of protein obtainable from the soluble fraction of lysates and maximize their solubility throughout the isolation procedure. βME included for those LBDs that have several cysteine residues; dithiothreitol can be substituted, but it tends to interfere more significantly with Ni-NTA binding. PMSF and Benzamidine-HCl are not always enough to stop proteolysis. If the protein is noticeably degraded during the preparation, the authors recommend addition of a commercial protease inhibitor cocktail (one is available from Boehringer Mannheim) and completing the purification quickly at 5°C.
2. Imidazole competes with proteins for the Ni sites on the affinity column. The imidazole concentrations in the washes and elution buffer should be determined experimentally for each protein. Ideally, one would like to maintain enough imidazole with the protein to minimize the binding of contaminating proteins to the Ni columns without compromising the LBD purification yields. In the initial preparations, one should keep all the fractions, and determine empirically the best imidazole concentrations based on the fractionation of the LBD. Imidazole has substantial 280 nm absorbance at these concentrations, and this must be accounted for in the blanks of UV spectroscopy.
3. The composition of this solution can be varied as necessary. These materials are included because they enhanced long-term stability of many LBDs. In some cases, the authors might use other solutions that help facilitate further chromatography on cation- or anion-exchange media.
4. Urea is used as the denaturant, because it interferes less with Ni-NTA columns.
5. In many cases, it is better to use several refolding buffers that reduce the urea concentration less precipitously. For example, it is helpful to go from 8 *M* urea to 0 *M* urea in 1-*M* increments. In any case, one must be careful to determine whether a substantial amount of protein precipitated on the column and failed to elute.
6. The authors have found that commercially available codon-biased cells can generate higher protein expression yields if the encoding gene contains many codons infrequently used by *E. coli* (e.g., Arginine AGA/AGG).
7. In some cases, the fractionation of the protein into soluble and insoluble portions of the lysate can be adjusted by controlling the rate of protein expression. Reducing the temperature at induction to 25°C, or using one-twenthieth or less IPTG, can increase the amount of LBD in the soluble fraction.
8. One should optimize the protein yields by varying induction time and examining the total lysate for expressed LBD using SDS-PAGE (as in **Fig. 1A**).
9. SDS-PAGE only shows if the protein has degraded to a smaller size, or if the concentration of the correctly sized LBD is diminished. To check if the protein is aggregating or oxidizing into higher molecular weight species, one may examine it by gel filtration (the authors use a 25 cm Superdex-75 column).

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7

Physical Structure of Nuclear Receptor–DNA Complexes

Scott A. Chasse and Fraydoon Rastinejad

1. Introduction

Nuclear receptors are organized into distinct functional domains, of which the most conserved is a 66-amino-acid DNA-binding domain (DBD) ***(1)***. This region, in some cases together with its C-terminal extension into the hinge region, imparts the receptor's ability to bind to target DNA sequences and form the appropriate cooperative homodimeric or heterodimeric complexes ***(2–6)***. Important advances in understanding DNA recognition has come from the direct visualization of the protein–DNA complexes, which can be achieved through X-ray diffraction studies ***(7–12)***, as well as through other structural and biophysical methods that directly probe the DNA-binding surface of nuclear receptors ***(13–17)***.

The crystal structures will probably continue to provide important new insights that further uncover how this large superfamily of transcription factors can discriminate among a set of highly related DNA target sequences. However, because crystal structures provide inferences and hypotheses that require experimental testing by mutational and biochemical studies, the authors have found it useful to couple this technique with a fluorescence-based equilibrium binding study, which allows one to test the effect of point mutations in the DBD on DNA-binding affinity and dimerization on DNA. Moreover, because the design of the optimal oligonucleotides and polypeptides are essential to the success of the crystallization experiments, the fluorescence studies discussed here are helpful in identifying the optimal interacting species suitable for study by X-ray crystallography.

This chapter presents detailed instructions for preparing large quantities of highly purified nuclear receptor DBDs and DNA duplexes for biophysical stud-

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ies, including both solution-equilibrium binding experiments and crystallization of receptor–DNA complexes. A description is included of specific purification and screening methods, which, based on experience, are likely to yield useful crystalline specimens for DBD–DNA complexes. However, a detailed description of crystallization methods and computational techniques used to solve macromolecular structures is beyond the scope of this chapter, and the reader is referred to more comprehensive volumes on these topics ***(18–21)***.

For measurements of equilibrium-binding constants, fluorescence polarization (FP) is a highly sensitive, rapid method that can be easily adapted to most protein–DNA systems ***(16,22–26)***. Unlike the more commonly used technique of electrophoresis mobility shift assays, FP measurements are made entirely in the aqueous phase; avoiding any complications or artifacts possibly introduced by the solid-phase support used in electrophoresis mobility shift assay. FP makes use of a fluorophore, such as fluorescein, which is covalently attached to the 5' or 3' of the target oligonucleotide. FP indirectly measures the tumbling rate of the fluorescent-labeled molecule. The unbound DNA tumbles more rapidly and has lower polarization than the larger DNA–protein complex. The rotational properties can be measured in terms of the anisotropy or, alternatively, the polarization of the FP signal as a function of increasing amounts of protein concentration. In this way, the technique allows the measurement of dissociation equilibrium constants extending from millimolar to picomolar levels under a wide variety of conditions.

2. Materials

2.1. Purification of Nuclear Receptor DNA-Binding Domains

One may use various *Escherichia coli* expression systems to produce the required proteins for cocrystallization and binding studies. However, a considerable amount of time-savings can be gained by using an expression system that produces a fusion-tag that facilitates purification of any DBD through affinity chromatography. Pharmacia's pGEX expression vectors produce N-terminal glutathione-*S*-transferase (GST) fusion tags that produce relatively easy scale-up and purification of these polypeptides. Depending on the choice of the pGEX vector and restriction sites, one may take advantage of specific protease digestion sites incorporated in the fusion construct, which allow removal of the tag following the affinity purification.

1. DBD expression vector: The cDNA coding sequence for a nuclear receptor DBD appropriately digested and ligated into the *Bam*HI and *Eco*RI sites of the pGEX-4T expression vector.

2. Luria-Bertani broth with ampicillin (LB + AMP): 10 g peptone, 5 g yeast extract, 10 g NaCl, pH to 7.4, bring to 1 L, autoclave; cool, add 1 mL 100 mg/mL AMP solution.
3. Lysis buffer: 25 m*M* Bis-Tris propane (BTP), pH 7.5, 300 m*M* NaCl, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* benzamidine-HCl.
4. Wash buffer: 25 m*M* BTP, pH 7.5, 400 m*M* NaCl.
5. Elute buffer: 25 m*M* BTP, pH 7.5, 100 m*M* NaCl, 10 m*M* glutathione.
6. Poros HS-20 high-performance liquid chromatography (HPLC) column or other strong cation exchangers.
7. HPLC buffer A: 25 m*M* BTP, pH 7.5, filtered.
8. HPLC Buffer B: 25 m*M* BTP, pH 7.5, 1000 m*M* NaCl, filtered.
9. FP buffer: 25 m*M* BTP, pH 7.3, 50 m*M* NaCl, 1 m*M* dithiothreitol.

2.2. Purification of DNA Suitable for Crystallization

1. Hamilton PRP-1 reverse-phase (RP) HPLC column, preparative size. RP-HPLC buffer A: 50 m*M* triethylammonium acetate, pH 6.0, filter to remove insolubles.
2. RP-HPLC buffer B: 50 m*M* triethylammonium acetate, pH 6.0, filter (as 2X stock) and add acetonitrile to 50% (v/v) final concentration.
3. Trifluoroacetic acid, 0.5% (v/v) solution.
4. Pharmacia Fast-Q anion exchange chromatography resin.
5. Q-column equilibration buffer: 20 m*M* Tris-HCl, pH 8.0.
6. Q-column elution buffer: 20 m*M* Tris-HCl, pH 8.0, 1 *M* NaCl.

3. Methods

3.1. Expression and Purification of DBDs

1. Transform the DBD expression vector into an *E. coli* BL21 expression host.
2. Prepare a starter culture by inoculating 50 mL LB + AMP solution with the transformed expression host and incubate with shaking for 15–24 h at 37°C.
3. Inoculate 1 L of LB + AMP with 10 mL of the starter culture and incubate with shaking at 37°C until the absorbance of the culture at 600 nm measures 0.7 (approx 2.5 h).
4. Induce protein expression by adding isopropyl-D-thioglucoside to 0.5 m*M* final concentration and incubate with shaking for 3 h at a reduced temperature of 30°C.
5. Harvest the cells by centrifuging for 20 min at 5000*g* and freeze at –20°C if desired (*see* **Note 1**).
6. Cell paste should be thawed on ice and kept cold throughout the remainder of the isolation procedures unless otherwise indicated.
7. Resuspend the cell paste completely into lysis buffer (8–10 mL/g cell paste).
8. Sonicate the cells thoroughly using three cycles or more (40 s/cycle followed by rechilling). Maintain the cell suspension temperature below 10°C.
9. Transfer the solution to ultracentrifuge tubes and centrifuge at 35,000*g* for 1 h.
10. Carefully decant the supernatant to a fresh tube, avoiding the last 5% because it contains contaminants.

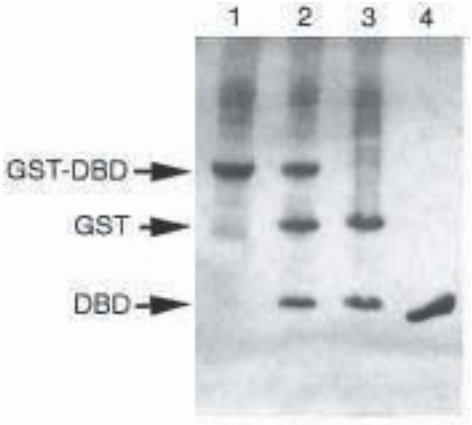

Fig. 1. SDS-PAGE with silver staining showing GST-RXR-DBD purified from the soluble fraction of *E. coli* via glutathione-Sepharose chromatography (*lane 1*), followed by increasing incubations of thrombin to proteolytically cut the fusion tag from the DBD (*lanes 2* and *3*). Because of the basic nature of nuclear receptor DBDs, the DBD can be readily purified from GST using a strong cation-exchange HPLC column (*lane 4*).

11. Load the solution onto a freshly equilibrated 10 mL bed of glutathione Sepharose 4B (Sigma) column and wash overnight with lysis buffer (>500 mL).
12. Wash the column with wash buffer until the absorbance at 260 nm is less than 0.05 (*see* **Note 2**).
13. Elute the purified protein from the column using elute buffer and fractionate the eluate into 2.5-mL fractions.
14. Measure the absorbance at 260 nm and 280 nm for each fraction in order to identify the fractions containing primarily protein as opposed to a DNA–protein mix. Combine those fractions containing no contaminating DNA.
15. Digest the GST-tag from the DBD using thrombin by first measuring the volume of the combined column fractions. Add bovine thrombin (Sigma, 900 NIH U/mL) at the concentration of 2 μL thrombin/mL protein, followed by incubation at room temperature for 6 h (*see* **Note 3**). **Figure 1** shows the expected result of thrombin cleavage for a GST fusion with a nuclear receptor DBD.
16. Isolation of the DBD from the GST tag and thrombin is achieved by loading the digested protein solution onto a strong cation exchange HPLC resin. The authors prefer POROS 20 HS because it can sustain higher flow rates and thus can speed purification without loss of resolution. However, other strong cation exchangers (such as Pharmacia's Mono-S) can be substituted, but run at a lower flow rate.
17. The following HPLC gradient is designed for purification of the DBD. The authors use a flow rate of 7.0 mL/min.

Time (min)	% Buffer B
0	0
9:00	17
29:00	50
29:30	100
34:30	100
35:00	0
45:00	0

18. Collect 7-mL fractions and identify the location of the DBD peak by monitoring the fractions at 280 nm. DBD should elute from the column as a single peak at approx 400 m*M* NaCl, and appear homogeneous by sodium dodecyl sulfate-polyacrylaide gel electrophoresis (SDS-PAGE) analysis (**Fig. 1**).

3.2. Design of Fluorescein-Labeled DNA

Several considerations must be made when designing the fluorescein-labeled DNA used in these experiments. First, the smaller the labeled DNA probe is in comparison with the DBD, the larger the increase in the anisotropy values upon protein binding. Therefore, the DNA should be designed to be as short as possible to accommodate the response element, but long enough to include 2–3 complementary base pairs at either end of the response element itself. These additional base pairs should be G:C base pairs to reduce end-breathing of the DNA. For a DBD that dimerizes on its response element, the labeled probe is likely to be approx 17–23 bp long. Both the unlabeled antisense strand and the fluorescein-labeled sense strand can be commercially obtained. The authors use ethanol precipitation to remove some contaminants from the DNA prior to annealing the strands.

3.3. Fluorescence Anisotropy Measurements

The high-sensitivity fluorescence measurements can be facilitated by the use of a dedicated fluorescence polarization system, such as the Beacon-2000 from Panvera (Madison, WI).

1. Anneal the labeled and unlabeled oligonucleotides by calculating their respective extinction coefficients and mixing in a 1:1.1 stoichiometric ratio (excess unlabeled strand) in a 2 mL screw cap tube, heating to 90°C in a water bath, and slow-cooling to room temperature overnight (*see* **Note 4**).
2. Dialyze freshly purified protein against FP buffer (*see* **Note 5**).
3. Concentrate freshly purified protein to 50 μ*M*.
4. Prepare a series of microcentrifuge tubes for a serial dilution of the protein over any desired range that covers the expected K_d (e.g., 10^{-6} *M*–10^{-9} *M*). Plan to have at least 150 μL sample remaining in each tube following the serial dilution.
5. Serially dilute the DBD solution.

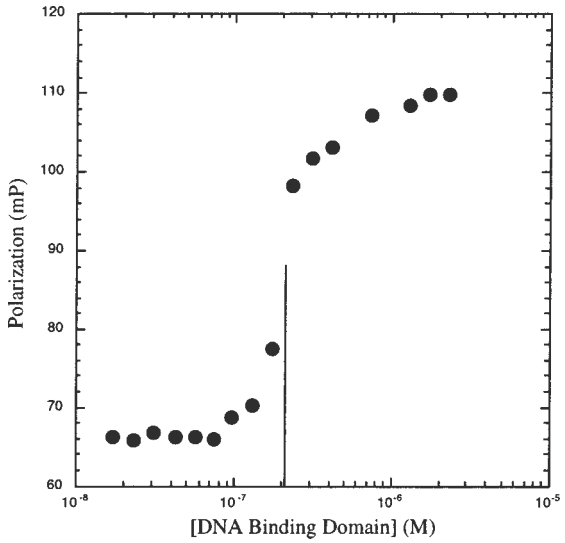

Fig. 2. Example of a fluorescence polarization study to determine the equilibrium binding of RXR-DBD to a fluorescein-labeled 15 bp duplex containing two tandem copies of AGGTCA with 1 bp spacing. The vertical line indicates the concentration required to achieve half-maximal binding.

6. Transfer 120 µL of each sample to individual, clean FP cuvets.
7. Using the "Batch Blank" program of the fluorescence polarimeter, measure the blank value of each sample (*see* **Note 6**).
8. Add 1.2 µL fluorescein-labeled DNA to each sample cuvet (*see* **Note 7**).
9. Gently mix each sample well using a pipet and allow to reach equilibrium at room temperature.
10. Measure each sample and record the polarization/anisotropy values (the instrument automatically subtracts the blank values from each sample).
11. Plot the anisotropy vs DBD concentration on a semi-log plot: The midpoint (50% binding concentration) can be measured directly. An example is shown in **Fig. 2** (*see* **Note 8**).

3.4. Purification of DNA Suitable for Cocrystallization with DBDs

The design and purity of the appropriate synthetic oligonucleotide is critical to the success of the crystallization. **Figure 3** shows how the DNA duplexes often make stabilizing end-to-end stacking interactions that give rise to the crystalline lattice. The DNA must contain the necessary recognition site; however, the flanking sequences and the total length can determine the success of cocrystallization trials. Because it is not possible *a priori* to design the ideal duplex size and flanking structure to produce the best diffracting crystals; the

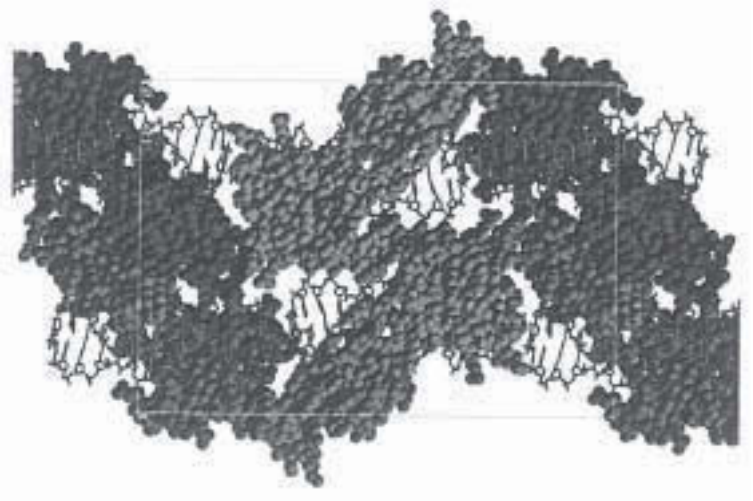

Fig. 3. The crystallographic packing interactions in the asymmetric unit (boxed) of crystals, containing the RXR DBD homodimer bound to a 15-bp DNA. End-to-end DNA stacking is a major determinant of crystallographic packing in this and many other protein–DNA complexes. As a result, the total size of the DNA and its terminal base structure is an important parameter in crystallization screens.

authors recommend using a number of alternative duplexes, all of which preserve the necessary internal sequence for DBD recognition, but which cover a reasonable size range (15–25 bp) and alternative terminal-base compositions.

For each synthetic oligonucleotide, one must extensively purify each of the two strands using RP-HPLC. In the first step, the authors purify the strand containing the 5' protecting dimethoxytrityl group (DMT). After removing the 5' protecting group (DMT) by acid hydrolysis, a second reverse-phase HPLC step helps further purify the oligos. In addition to these chromatographic steps, anion-exchange chromatography (Fast-Q) is useful for concentrating DNA, removing the protecting group, and exchanging out the HPLC solvents.

1. Commercially available 1-μmol size synthesis leaving the 5'-DMT on the oligo facilitates purification. Each 1 μmol synthetic DNA yields about 40–50 optical density units (at 260 nm) of purified material. These syntheses arrive in a lyophilized state. The DMT-on HPLC is normally completed within the same day to prevent acid detritylation in the low-pH buffer used for HPLC.
2. Dissolve the DNA in 3–4 mL RP-HPLC buffer A, microcentrifuge at 10,000*g* for 10 min to remove insolubles from the DNA.
3. Inject analytical amount of sample (one-tenth or less of total sample) onto the HPLC PRP-1 column. The DMT-on HPLC step is run with this program at a flow rate of 2.5 mL/min.

Time (min)	% Buffer B
0	0
3:00	30
35:00	55
35:30	100
42:00	100
43:00	0
60:00	0

The DMT-on DNA should be the biggest peak, eluting between 30 and 55% buffer B. If the HPLC results in good resolution of the product, inject the remaining sample over 3–6 additional runs.

4. Dilute peak fractions with equal amounts of deionized water to lower the buffer concentration.
5. Pour a 2-mL column of Pharmacia Fast-Q (anion-exchange resin). Flow 5–10 column volume equilibration buffer. Load diluted peak fractions from HPLC onto column, and wash with 10 column volume equilibration buffer.
6. Wash column with 5 column volume pure water. Immediately load a 0.5% solution of trifluoroacetic acid onto column, wait 15 min for acid-removal of DMT group.
7. Wash with 10 column volume equilibration buffer.
8. Using elution buffer (which contains 1 *M* NaCl), collect 1–2 mL fractions. Prepare 1:100 dilutions (by removing 10 μL of each fraction and add 990 μL H_2O) for UV spectrum analysis, to identify peak fractions. Pool the original peak fractions together (total 4–6 mL) for DMT-off HPLC, which follows.
9. Run the PRP-1 HPLC column with the following program at 2.5 mL/min flow rate:

Time (min)	% Buffer B
0	10
35:00	30
35:30	100
42:00	100
43:00	10

10. Again, inject one-tenth or less of your sample in an analytical run. The DMT-off product should elute between 10 and 35% B.
11. Inject remainder of the sample over 4–6 runs. Pool all fractions together and add equal volume water to lower ionic strength.
12. Pour fresh 2 mL Fast-Q column; equilibrate with Q-resin equilibration buffer.
13. Load pooled fractions, wash with 10 column volume equilibration buffer.
14. Use elution buffer to collect sharp (1 mL) fractions, identify peak DNA fractions using 1:100 dilutions as before (*see* **step 9**); freeze at –20°C until ready to anneal with complementary strand.
15. To anneal the duplex, calculate the molar extinction coefficients for each strand based on the base composition and make a 1:1 complex. Combine the strands in a screw-top Eppendorf tube and place in a beaker containing water heated to

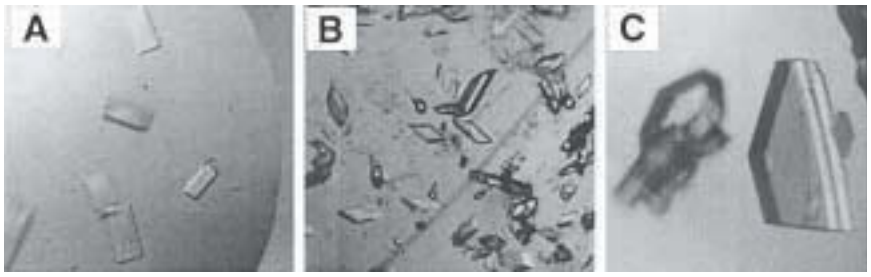

Fig. 4. Examples of DBD-DNA cocrystals obtained using the screening procedure based on the precipitant PEG and various concentrations of monovalent and divalent ions described in the text at pH 6-8. **(A)** Crystals of RXR DBD homodimer bound to 15-bp DNA. **(B)** Crystals of RXR-RAR DBD heterodimer bound to 15-bp DNA. **(C)** Crystals of RevErb DBD bound to a 20-bp DNA.

90°C. Place the beaker inside a Styrofoam box, seal, and allow to cool slowly to room temperature overnight.

3.5. Crystallization Screens for Nuclear Receptor–DNA Complexes

Combine the appropriate amount of duplex DNA and purified DBD to produce a final concentration of 0.1 m*M* DNA/0.1 m*M* each DBD when concentrated to 300–400 μL. Concentrate to this volume using a Centricon-10 (Amicon) device, replacing the lost volume three times with the final buffer (typically, 20 m*M* of a buffer at pH 6.0, 7.0, or 8.0, 50 m*M* NaCl). The authors carry out three exchange steps in which 90% of the original volume is replaced per step.

To screen for crystallization, the authors recommend the use of commercial (Hampton) screens I and II. However, in the authors' experience, a systematic search around the conditions of buffer, $MgCl_2$, salt, and polyethylene glycol (PE6) concentrations, shown below, often yields better initial results (*see* **Fig. 4** for some examples of DBD–DNA crystals obtained using this screen). In either case, once microcrystals are observed in the screen, a more focused search around the initial conditions is recommended to converge on single larger crystals suitable for data collection.

1. Buffer/pH conditions (at 25 m*M*):
   a. MES, pH 6.0.
   b. BTP, pH 7.0.
   c. Tris, pH 8.0.
2. $MgCl_2$ concentrations:
   a. 5 m*M*.
   b. 50 m*M*.

3. NaCl or NH_4Cl concentrations:
   a. 50 m*M*.
   b. 200 m*M*.
4. Polyethylene glycol:
   a. 5–35% PEG-1000.
   b. 5–35% PEG-3350.
   c. 5–35% PEG-8000.

Set up vapor-diffusion hanging drops (using standard crystallization, 24-well Linbro plates), in which a systematic crystallization screen is undertaken, using combinations of these reagents. For example, one plate can be used to systematically test the effect of pH on crystallization. In this plate, the first row may contain 25 m*M* Tris, pH 8.0, 5 m*M* $MgCl_2$, 200 m*M* NaCl, with increasing amounts (10, 12, 14, 16, 18, 20%) of the precipitant, PEG-8000. Subsequent rows differ only in the choice of buffers and pHs. Hanging drops are convenient for screening, and should be made using 2 μL of the protein–DNA solution and 2 μL of the well solution. Crystallization plates should be set up in vibration-free, controlled temperature environments and examined every few days for crystal growth.

3.6. Preliminary Analysis of Cocrystals

The analysis of crystals involves three steps prior to data collection. In the first step, one does a visual inspection to determine the size and suitability of the crystal. Ideally, the size of the crystals should be >0.15 mm in each of the three axes. The crystals may be transferred to successive fresh drops containing only the crystallization solutions. These steps will wash any protein and DNA components on the outside of the crystals. The authors transfer crystals using small commercially available nylon loops attached to stainless steel pins (available from Hampton Research). After the third wash, the crystals may be dissolved in a minimal volume of SDS-PAGE sample buffer and its macromolecular components are examined by silver-staining the gel. This type of biochemical analysis will help establish that the correct species has in fact been crystallized, because DNA or protein alone crystals must be ruled out. Having established the protein–DNA composition, one follows by mounting the crystals in a thin glass capillary containing a small volume of mother liquor for hydration. The X-ray diffraction pattern should clearly reveal whether the crystals are useful for high-resolution data collection.

4. Notes

1. Frozen cell paste may remain frozen and the fusion protein will remain safe for indefinite lengths of time. However, it is not necessary to freeze the cell paste as part of the purification.

2. DBDs can bind nonspecifically to chromosomal DNA. Therefore, it is necessary to monitor the column flowthrough at 260 nm to confirm at what point the contaminating DNA has been washed from the column prior to protein elution.
3. Thrombin, at the prescribed rate, will digest any reasonable concentration of GST-retinoid X receptor (RXR)-DBD fusion protein to completion in 6 h. However, it is prudent to optimize the digestion parameters (time, thrombin rate) for each unique fusion protein.
4. The labeled oligonucleotide should be slightly in excess to ensure that 100% of the labeled DNA is in duplex form. Unduplexed, labeled oligonucleotide will not bind appreciably to DBD, but may inappropriately contribute to the total FP signal.
5. The purified DBD will elute from the HPLC column at a salt concentration of roughly 400 m*M* NaCl. To expedite dialysis of the protein, to contain the desired salt concentration of 50 m*M*, measure the volume of the protein solution and dialyze this volume against 10 vol of a 25 m*M* BTP, 1 m*M* DTT solution at 4°C. After at least 8 h, exchange the dialysis buffer with FP buffer and dialyze overnight.
6. The Beacon® 2000 System should be programmed as follows: Read Mode = Static, Blank Type = Batch, Blank Delay = 0, Sample Delay = 0, Number of Average Reads = 10, Single Point Temperature = 25°C (or room temperature), Control Type = Autorange.
7. The concentration of the fluorescent-labeled DNA should be as low as possible (below the expected K_d) and still yield stable, reproducible intensity/anisotropy values. For a fluorescein-labeled DNA probe, the sensitivity of the Beacon 2000 is in the n*M* to p*M* range. The actual concentration should be empirically determined for each individual molecular system. Finally, a stock concentration should be chosen so that a very small (e.g., one-hundredth sample vol) addition of labeled DNA solution will not affect the protein concentration significantly.
8. It is also important to plot the intensity vs the DBD concentration to determine whether the intensity has significantly (± >15% of the mean) changed over the relevant protein concentrations.

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8

Isolation of Steroid-Regulated Genes from the Uterus by mRNA Differential Display

Sushma Kumar, Maarit Angervo, Milan K. Bagchi, and Indrani C. Bagchi

1. Introduction

Steroid hormones, estrogen, and progesterone, promote extensive cell proliferation, differentiation, and remodeling in all compartments of the uterus during pregnancy *(1–5)*. These hormones orchestrate the entry of the fertilized ova into the uterus, prepare the uterus for embryo implantation, and maintain an environment conducive to the growth and development of the implanted embryo. The cellular actions of these hormones are mediated through specific intracellular receptors. These receptors function as ligand-inducible transcription factors *(6–8)*. It is generally believed that the cellular events leading to the establishment and maintenance of pregnancy are mediated through the expression of specific steroid-regulated genes in the uterus. The identification of these steroid-regulated genes is crucial for understanding the molecular and cellular processes that control uterine growth and differentiation during pregnancy.

In an attempt to identify genes that mediate estrogen and progesterone action in the uterus during pregnancy, the authors employed the mRNA differential display technique (polymerase chain reaction differential display [DD-PCR]) devised by Liang and Pardee *(9)*. The invention of the DD-PCR method, which directly compares the expression profiles of cDNAs obtained from two different pools of mRNA, has simplified gene identification by differential expression cloning *(9–13)*. It has many advantages over a differential screening method based on subtractive hybridization. The DD-PCR method allows one to display and analyze the majority of the mRNAs expressed in an eukaryotic cell by using only a few micrograms of total RNA, which gives it a crucial advantage over other differential screening methods in analyzing minute

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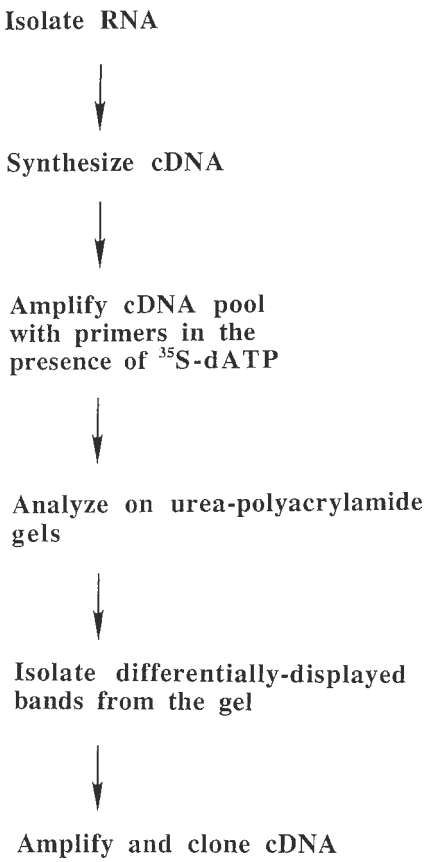

Fig. 1. A schematic representation of the DD-PCR method.

quantities of human tissue specimens. Another major advantage of the DD-PCR method is that simultaneous identification of up- and downregulated genes can be achieved in the same experiment, and that multiple RNA populations can be compared in the same gel. The authors have successfully used this technique to isolate several steroid-regulated genes from rodent or human uterus ***(14,15)***. This chapter describes the steps involved in isolation of steroid-regulated genes from rodent endometrium.

The major steps in the isolation of such genes from uterus are described in **Fig. 1**. Briefly, the method involves the reverse transcription of the cellular mRNAs using oligo-deoxythmidine primers anchored to the 5'-end of the poly-adenylic acid tail, followed by amplification of the resulting cDNAs by PCR, using a second oligonucleotide primer of random sequence. The cDNAs are labeled with ^{35}S during amplification. The amplified cDNA subpopulations

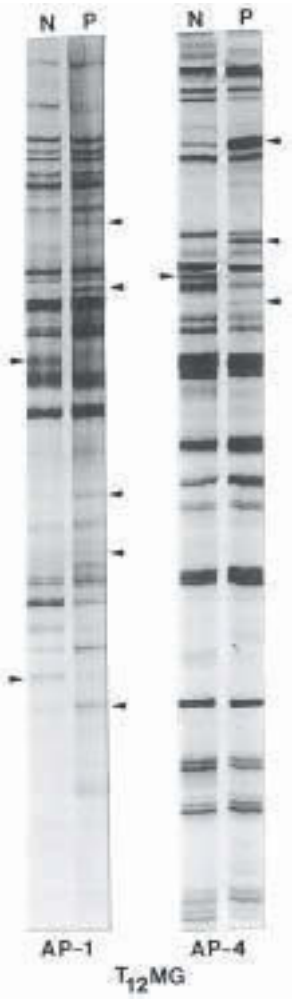

Fig. 2. Profiles of the differentially expressed mRNAs in nonpregnant (*lane N*) and pregnant (*lane P*) rat uterine tissues.

representing 3' termini of mRNAs as defined by this pair of primers are then analyzed in adjacent lanes on a DNA sequencing gel. The differential expression of mRNAs can be readily detected by visually scanning an autoradiogram. **Figure 2** shows a typical mRNA DD gel, which the authors routinely obtain in this laboratory. By changing primer combinations, as many as 15,000 individual mRNA species from a mammalian cell may be visualized. The differentially displayed bands, representing potential differentially expressed mRNAs, are recovered from the gels, and, following further PCR amplification, are

subcloned and sequenced to determine identity. The differential expression of isolated cDNAs is then further confirmed by Northern blot analysis using the original mRNA pools. Finally, the steroid hormone regulation of isolated cDNAs is examined by treating ovariectomized animals with exogenous estrogen and progesterone, isolating mRNAs from tissues of treated animals, and performing Northern blot analysis using a radiolabeled cDNA probe of interest. Additional information related to this subject is described in **Notes 1–8**.

2. Materials

1. RNase-free water (water treated for 1 h with 0.1% diethylpyrocarbonate [DEPC], and then autoclaved).
2. Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene).
3. Deoxyribonucleoside triphosphate (dNTP) (Perkin Elmer).
4. DNaseI, RNase-free (Stratagene).
5. T4 DNA ligase (Promega).
6. *Escherichia Coli* JM 109 competent cells (Promega).
7. $\alpha[^{35}S]$ deoxyadenosine triphosphate (dATP) (1200 Ci/mmol) (Amersham).
8. *Taq* DNA polymerase (Perkin Elmer).
9. Formamide gel loading buffer: 95% formamide, 10 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0, 0.09% xylene cyanol, 0.09% bromophenol blue.
10. 20 mg/mL Glycogen (Boehringer-Mannheim).
11. Whatman 3 MM filter paper.
12. 5X TBE buffer (for 1 L): 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA, pH 8.0.
13. Agarose.
14. Acrylamide: bisacrylamide (19:1) (Bio-Rad).
15. TEMED (Bio-Rad).
16. Kodak X-ray film.
17. Monotec autoradiography cassettes (VWR).
18. Saran wrap.
19. 1.5-mL Microtubes (Sarstedt).
20. 0.5-mL PCR tubes (Perkin Elmer).
21. QIAEX II gel extraction kit (Qiagen).
22. Pin Point Xa1T vector system (Promega).
23. Agar plates.
24. Duralon nylon membrane (Stratagene).
25. Salmon sperm DNA (Sigma).
26. 20X Standard sodium citrate (SSC) buffer: 175.3 g NaCl, 88.2 g Na citrate, 800 mL of water. Adjust to pH 7.0 with a few drops of 10 *N* NaOH, adjust volume to 1 L with water.

2.1. Special Equipment

1. Polytron homogenizer with 7-mm diameter probe (Brinkman).
2. Sequencing Unit (Bio-Rad).

3. Power supply (Fisher Biotech).
4. Stratalinker UV Crosslinker (Stratagene).

3. Methods

In a typical DD-PCR experiment, the authors first extract total RNA from nonpregnant and pregnant rodent uteri. RNA samples are freed of DNA after treatment with DNaseI. 2 μg DNA-free total RNA is then reverse-transcribed with MMLV-RT in the presence of 1 μ*M* of T_{12}MA, T_{12}MC, T_{12}MT, or T_{12}MG primer (where T represents thymidine and M is a degenerate mixture of adenosine, A, cytosine, C, and guanosine, G), to synthesize cDNAs. One tenth of the cDNA reaction is then used in a PCR amplification reaction containing 2 μ*M* each of four dNTPs, 10 μCi of ^{35}S-dATP, 2 primers: 1 μ*M* of one of the four T_{12} oligonucleotides and 0.2 μ*M* of one of the five arbitrary decamers, AP-1 (5'-AGCCAGCGAA-3'); AP-2 (5'-GACCGCTTGT-3'); AP-3 (5'-AGGTGACCGT-3'); AP-4 (5'-GGTACTCCAC-3'); AP-5 (5'-GTT GCGATCC-3'). The primers are obtained from Genhunter.

3.1. RNA Preparation

Uteri were snap frozen in liquid nitrogen, weighed, and stored at –70°C, until further use. The method describes isolation of RNA from 500 mg tissue.

1. Prepare denaturing solution in RNase-free water by adding 4 *M* guanidinium isothiocyanate, 0.02 *M* Na citrate, and 0.5% sarcosyl. The solution can be prepared in advance and stored at 4°C.
2. Prepare homogenization buffer just before RNA isolation by adding 7 μL β-mercaptoethanol to 1 mL denaturing solution. Use 5 mL denaturing solution for 500 mg tissue. If less or more tissue is needed, the amount of denaturing solution can be adjusted accordingly. Homogenize the tissue using Polytron homogenizer.
3. Transfer the homogenate to a microcentrifuge tube. Add 3 *M* Na acetate, pH 4.0 (volume of Na acetate added is one-tenth of the homogenization buffer used) prepared in DEPC H_2O to the homogenate.
4. Add 5 mL of water saturated phenol, pH 5.5.
5. Add 1 mL chloroform:isoamyl alcohol, cap tightly, and vortex vigorously.
6. Centrifuge the mixture at 14,000 rpm for 5 min. Two phases should be clearly visible.
7. Carefully transfer the aqueous phase containing the RNA to another microcentrifuge tube.
8. Add 2.5 μL 20 mg/mL glycogen and 5 mL isopropanol. Mix by inverting the tube.
9. Centrifuge the sample at 14,000 rpm for 30 min.
10. Remove the supernatant and wash the pellet with 5 mL 85% ethanol in DEPC water. Centrifuge at 14,000 rpm for 10 min.
11. Discard the supernatant carefully and air-dry the pellet.

12. Resuspend the RNA pellet in 500 μL DEPC-treated water. Incubate at 55°C for 10 min.
13. Remove an aliquot, dilute it with distilled water for spectrophotometric measurement. Measure the optical density at 260 and 280 nm to check the quantity and quality of RNA.
14. Store the rest of the RNA at –80°C.
15. Examine the quality of the RNA by electrophoresis of a sample on a denaturing formaldehyde agarose gel. Total RNA typically yields bright 28S and 18S ribosomal RNA bands at approx 4.5 and 1.9 kb upon ethidium bromide staining.

3.2. DNase Treatment of Total RNA

Add to the microcentrifuge tube in the following order:

1. 50 μg Total RNA, 5.0 μL 10X reaction buffer (400 m*M* Tris-HCl, pH 7.5, 60 m*M* $MgCl_2$, 20 m*M* $CaCl_2$), and 1 μL DNaseI (10 U/μL), to a total volume of 50 μL.
2. Mix and incubate at 37°C for 30 min.
3. Add 50 μL phenol, pH 5.5:chloroform (3:1). Vortex for 30 s.
4. Incubate for 10 min on ice.
5. Centrifuge the tube at 14,000 rpm for 10 min at 4°C.
6. Transfer the aqueous phase to a clean microcentrifuge tube. Add 1 μL glycogen (2 mg/mL), 5 μL of 3 *M* Na acetate, and 200 μL 100% ethanol.
7. Place the tube at –80°C for 1 h (for maximal precipitation of RNA, the samples can be left overnight at –70 C).
8. Centrifuge the RNA sample for 30 min (14,000 rpm) at 4°C.
9. Remove the supernatant and wash the pellet once with 200 μL of 85% ethanol (in DEPC-treated water). Spin for 10 min.
10. Air-dry the pellet for 10–15 min.
11. Dissolve the pellet in 1.5 μL DEPC-treated water for each 2 μg of starting RNA.
12. Store RNA sample at –80 C.
13. Run 2–3 μg of purified RNA on an agarose gel to check the integrity of the sample.

3.3. First Strand cDNA Synthesis

For each RNA sample, label four 0.5-mL capacity Perkin Elmer tubes as G, A, T, and C. Each tube represents a 3' degenerate oligo-deoxythymidine primer, including T_{12}MA, T_{12}MC, T_{12}MT, or T_{12}MG. Set up the following reaction on ice.

5X RT buffer	4.0 μL
dNTP (250 μ*M*)	1.6 μL
Total RNA	2 μg
T_{12} primer	2.0 μL
Water	X μL to make the final reaction volume 19 μL

5X RT buffer: 125 m*M* Tris-HCl, pH 8.3, 188 m*M* KCl, 7.5 m*M* $MgCl_2$, 25 m*M* dithiothreitol.

In this laboratory, when multiple cDNAs are prepared, the authors make master mixes to avoid pipeting error, e.g., if cDNA is to be prepared for four samples, the mixes will be prepared for five tubes. The following calculations have been done for making mix for five tubes.

Mix	Primer (μL)	Buffer (μL)	dNTP (250 μ*M*) (μL)	Add/tube (μL)
Mix 1: T_{12} MG primer	10	20	8.0	7.6
Mix 2: T_{12} MC primer	10	20	8.0	7.6
Mix 3: T_{12} MA primer	10	20	8.0	7.6
Mix 4: T_{12} MT primer	10	20	8.0	7.6

1. Add RNA (2 μg) and water in a total volume of 11.4 μL to each tube. The final volume for each tube should be 19 μL.
2. Incubate the tubes at 65°C for 5 min.
3. Transfer the tubes to 37°C for 10 min.
4. Add 1 μL of MMLV-RT to each tube.
5. Mix the contents by gentle pipeting.
6. Spin the tubes briefly in a microcentrifuge.
7. Transfer the tubes to 37°C water bath for 50 min.
8. Terminate the reaction by incubating the tubes at 95°C for 5 min.
9. Place the tubes on ice.
10. Spin the tubes briefly in a microcentrifuge.
11. Store all cDNA tubes at –20°C until further use.
12. Use one tenth of the cDNA reaction (2/20 μL) in DD-PCR reaction.

3.4. DD-PCR

The authors recommend that combinations of 3'-T12 primers and 5'-AP series of primers, available from Genhunter, be used in the DD-PCR reactions. If, for example, one starts with six samples and tries the combination of one of the four 3'-T12 MN primers with five 5'-AP-1–AP-5 primers, the total number of tubes will be 30. Therefore, PCR master mix can be prepared for 32 tubes.

Set up PCR reactions at room temperature. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin.

1. Prepare enough master mix. The table below gives volumes to be used for 32 tubes.

Component	Per reaction (μL)	For 32 reactions (μL)
Water	9.2	294.4
10X PCR buffer	2.0	64.0
dNTP (25 μ*M*)	1.6	51.2
T12MN (10 μ*M*)	2.0	64.0
^{35}S-dATP (1200 Ci/mmol)	1.0	32.0
Amplitaq polymerase	0.2	6.4
	16.0	512.0

2. Mix the contents of the tube by vortexing and briefly spin in a microcentrifuge.
3. Aliquot 16 µL PCR mix into each tube.
4. Add 2 µL reverse transcribed cDNA (from **Subheading 3.3.**) into each tube. The T12 primer that is used in the reverse transcription reaction in **Subheading 3.3.** should be used for PCR mix.
5. Add 2 µL AP primer. This will give a final volume of reaction mixture to 20 µL.
6. Mix well by pipeting up and down.
7. Begin thermal cycling using the following amplification cycles.

40 Cycles:	30 s	94°C
	2 min	40°C
	30 s	72°C
1 Cycle:	5 min	72°C

Final step: Cool down and maintain at 4°C.

8. Mix 4 µL of each sample with 2 µL loading dye and incubate at 80°C for 2 min before loading onto a 6% DNA sequencing gel.

3.5. Electrophoresis

The authors routinely use Bio-Rad sequencing gel apparatus to run the differential display gels.

1. Pour a 6% denaturing polyacrylamide/8 *M* urea gel in 1X TBE buffer. The ratio of acrylamide to bisacrylamide should be 19:1.
2. Let the gel polymerize for at least 2 h before using. The gel can also be poured and polymerized a day in advance, covered with Saran wrap, and kept at room temperature.
3. Prerun the gel at 60 W for 1 h or until the temperature reaches 50°C. Rinse wells of the gel with buffer in order to flush out all the urea.
4. Load samples onto the gel and perform electrophoresis at 60 W for approx 4 h or until the blue dye (xylene cyanol) runs to the bottom of the gel.
5. After electrophoresis, remove the notched plate and transfer the gel on a piece of 3MM paper. Cover the gel with plastic wrap and dry it under vacuum on a gel dryer at 80°C for 1 h.
6. Peel off the plastic wrap and expose the gel to a X-ray film for 24–72 h at room temperature. Be sure to mark the gel with autoradiographic markers (Stratagene), so that the orientation of the film can be easily determined.

3.6. Recovery of DNA Fragments from Dried Polyacrylamide Gels

1. After developing the film, precisely superimpose the autoradiogram on the dried gel and cut out the gel areas that represent differentially expressed bands.
2. Mark the differentially expressed bands on the film by poking holes through the film and the gel beneath.
3. Cut out the located bands with a clean razor blade.

4. Place each of the cut-out bands from the dried gel, along with 3MM paper in microcentrifuge tubes. Add 100 µL water to the tubes and incubate for 10 min at room temperature.
5. Boil the tubes with cap locks on them for 15 min.
6. Microcentrifuge the tubes for 2 min to remove the gel slices and the paper debris.
7. Transfer the supernatant to a new microcentrifuge tube.
8. Add 2 µL glycogen (20 mg/mL), 10 µL 3 *M* Na acetate, and 450 µL 100% ethanol.
9. Place the tubes at –70°C overnight.
10. Centrifuge for 15 min at 14,000 rpm at 4°C to pellet the DNA.
11. Discard the supernatant and rinse the pellet with 200 µL ice-cold 85% ethanol.
12. Centrifuge at 14,000 rpm for 10 min at 4°C.
13. Air-dry the pellet, then dissolve in 10 µL water.

3.7. Amplification of DNA Eluted from Gel

Amplification of the DNA eluted from the gel should be done using the same primer set and PCR conditions as in **Subheading 3.4.**, except that 250 µ*M* dNTP stock is used instead of 25 µ*M*, and no radioisotope is added in the reactions. Use 4 µL cDNA (*see* **Subheading 3.6.**) for amplification and save the rest of it at 4°C.

1. Prepare the master mix for amplification of 10 samples as follows:

Component	Per reaction (µL)	For 10 reactions (µL)
10X PCR buffer	4.0	40.0
dNTP (250 m*M*)	3.2	32.0
T12MN primer	4.0	40.0
Water	20.4	108.0

   Add 4.0 µL cDNA and 4.0 µL AP primer to each tube. Finally, add 0.4 µL AmpliTaq enzyme to each tube. For convenience, one may prepare a master mix containing 4.0 µL enzyme and 96 µL water. Add 10 µL/tube.
2. Perform PCR amplification as described in **Subheading 3.4., step 7**.
3. Load 30 µL PCR sample on a 1% agarose gel for analysis.
4. If the first attempt at amplification fails, then an increased amount of first-round PCR product may be used as a template in a 40-cycle reamplification reaction.
5. Check that the size of the amplified DNA matches with the expected size of the band on the denaturing acrylamide gel.
6. Cut the band out of agarose gel and proceed for DNA extraction using QIAEX II kit. Gel slices can be frozen at –20°C until ready to extract DNA.

3.8. Subcloning and Nucleotide Sequence Analysis of Isolated cDNA

1. DNA is extracted from the gel slice using QIAEX II gel extraction kit (Qiagen) following manufacturer's specifications.

2. Dissolve the purified DNA in 10 μL water.
3. Subclone the DNA into Pin Point vector (Promega). The authors generally use the following protocol for ligation reaction:
   a. 1 μL: 10X Ligation buffer (300 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 100 m*M* dithiothreitol, 10 m*M* ATP).
   b. 3 μL: Purified DNA.
   c. 1 μL: Pin Point Vector (50 ng/μL).
   d. 1 μL: T4 DNA Ligase (1 U/μL).
   e. Water to a final volume of 10 μL.
   f. Incubate the reaction overnight at 15°C. Transform 2 μL ligation reaction into JM 109 competent cells.
4. After subcloning of isolated cDNAs, perform nucleotide sequencing (generally, this can be done at a DNA sequencing core facility) in order to determine the identity.

3.9. Confirmation of Differential Expression of the Isolated Clone by Northern Blot Analysis

It is necessary to confirm the differential expression of the isolated cDNA by using it as a probe in Northern blot analysis. For Northern analysis, 5 μg of polyadenylic acid plus mRNAs, isolated from nonpregnant and pregnant rodent uteri, are separated by formaldehyde agarose gel electrophoresis ***(16)*** and transferred to Duralon membrane (Stratagene). After transfer, the membranes are crosslinked using Stratalinker UV Crosslinker (Stratagene). Blots are prehybridized in 50 m*M* $NaPO_4$, pH 6.5/5X SSC/5X Denhardt's/50% formamide/0.1% sodium dodecyl sulfate (SDS) and 100 μg/mL salmon sperm DNA for 4 h at 42 C. Hybridization is carried out overnight in the same buffer, containing 10^6 cpm/mL ^{32}P-labeled cDNA probe. The filters are washed twice for 15 min in 1X SSC/0.1% SDS at room temperature, then twice for 20 min in 0.2X SSC/0.1% SDS at 55°C, and the filters are exposed to X-ray films for 24–72 h.

3.10. Steroid Hormone Regulation of Isolated cDNAs

In order to determine if an isolated cDNA is regulated by steroid hormones, experiments are performed using ovariectomized rats or mice. Two weeks after ovariectomy, animals are injected subcutaneously with either sesame oil (vehicle) or estrogen or progesterone. Typically, a single dose of the hormone is administered, but, in some cases, steroids are administered for up to 3 d in order to maximize the effect of the hormones on gene expression. The animals are killed 24 h after last injection, uteri are isolated, mRNAs are prepared from the tissue and are subjected to Northern blot analysis, as described in **Subheading 3.8.**

4. Notes

1. The DD-PCR method allows one to display and analyze the majority of the mRNAs expressed in an eukaryotic cell by using only 2 μg of total RNA. This ability gives it a crucial advantage over other differential screening methods in analyzing minute quantities of human tissue specimens. The authors have also successfully used this method to isolate cDNAs from human endometrial biopsies that are differentially expressed at the proliferative vs secretory phase of the menstrual cycle.
2. A major problem of DD-PCR is the isolation and amplification of spurious cDNAs that are present as contaminants in the differentially displayed band. To weed out these irrelevant clones, it is critical to confirm the differential expression of the isolated cDNA by Northern blotting. Following isolation and amplification, the cDNA fragment is radiolabeled with ^{32}P (by end-filling), and employed to probe Northern blots of total RNAs isolated from the uteri. The cDNA fragments exhibiting differential expressions in Northern blots are then subcloned into PinPoint vector (Promega) for sequencing and identification.
3. The authors suggest that duplicate DD-PCR reactions should be performed. Only bands indicating differential gene expression in duplicates should be isolated and processed for further characterization. This would limit the number of false positives arising from random fluctuations in individual reactions.
4. The authors strongly suggest that control DD reactions lacking reverse transcriptase should be run to eliminate signals that arise from genomic DNA contaminants in the mRNA pools.
5. Another shortcoming of the DD-PCR method is that the nucleotide sequence of isolated cDNAs often exhibit no homology to nucleotide sequences in the Genbank. This is because DD-PCR method amplifies the 3' end of the cDNAs, which, in many cases, correspond to the 3'-untranslated region. To alleviate this problem, the authors suggest that a longer cDNA of the clone be isolated from a cDNA library that is constructed from the tissue under investigation.
6. The authors generally prefer to isolate and analyze only those bands that represent cDNA fragments longer than 350 bp. Bands corresponding to cDNA fragments shorter than 350 bp often fail to provide adequate information about DNA sequencing, and tend to yield weak signals in Northern blot analysis.
7. Silicone-coated glass plates should be used to run the display gels, which helps the transfer of the gel to the paper following electrophoresis. Allow the glass plates to cool down to room temperature before attempting to separate the glass from the gel. A clean blade should be used for cutting out each band from the gel. Even a small contamination in the blade can lead to the amplification of the wrong cDNA.
8. In order to confirm steroid hormone regulation of isolated cDNAs, the authors recommend that additional experiments using antihormones should be performed. The antiprogestin RU486 or the antiestrogen ICI 182,780 counteracts the cognate hormonal pathway, by binding directly to the hormone receptor, and by

impairing its gene regulatory activity ***(17,18)***. In previous studies, the authors have observed that treatment of rats with a single injection of RU486 ***(19,20)*** or ICI 182,780 ***(14,21)***, abolishes progesterone- or estrogen-dependent gene expression within 24 h of treatment.

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9

Identification of Nuclear Hormone Receptor Homologs by Screening Libraries with Highly Degenerate Oligonucleotide Probes

Bruce Blumberg

1. Introduction

Orphan nuclear receptors have been identified using a variety of methods over the years. The first were identified by low-stringency hybridization using known receptors as probes. This strategy has been successful because members of the steroid receptor superfamily contain a conserved DNA-binding domain and share regions of similarity in the ligand-binding domain. These conserved regions may also be used to design polymerase chain reaction primers that have been used to identify new receptors, primarily members of known families. The recent explosive increase in DNA sequences from EST and genomic sequencing projects has also allowed the identification of new family members. The *Caenorhabditis elegans* genome has recently been sequenced and shown to contain a large variety of putative nuclear receptor genes, some of which may be represented in mammalian genomes. The question remains of how to identify potentially highly divergent mammalian homologs. One possibility is to wait until such sequences appear in the rapidly growing sequence databases from rodent and human genome projects. This method has been used to identify a novel member of the steroid receptor superfamily ***(1–3)*** and may ultimately result in the identification of others. For those who do not wish to wait, or who work on model organisms whose genome projects are not well advanced (e.g., *Xenopus*), there is no substitute for directly isolating the relevant cDNAs.

Polymerase chain reaction-based methods require two oligonucleotides. Designing two appropriate sequences may not always be possible because either sequence information is lacking or there is insufficient sequence conser-

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vation. This can be overcome by screening with a single oligonucleotide whose sequence is derived from amino acids in a region conserved in sequence among members of a gene family. Various oligonucleotide-screening strategies have been employed for this purpose in the past, including "guessmers," in which the wobble position of each codon is derived from a codon frequency table for the species in question, and the substitution of inosine in the wobble position, which may allow multiple types of sequences to be detected. Each of these methods introduces a bias that may result in an unsuccessful screen. The author prefers the use of oligonucleotides that represent all possible codons for the amino acids to be matched. These fully degenerate oligonucleotides are guaranteed to hybridize precisely to the target sequence and will always result in successful screens if a few precautions are taken.

The most serious problem with using highly degenerate oligonucleotides to probe blots results from the inability to predict which sequence, out of a family of sequences, is actually hybridizing to the target. Since the G:C content of each differs, one cannot easily pick a hybridization and washing temperature that minimizes the number of false hybridization signals. This problem can be overcome by the use of 3.0 *M* tetramethylammonium chloride (TMAC) in the washing buffer ***(4–6)***. In this method, one hybridizes at low stringency, and washes at high stringency, reducing the number of false positives. 3.0 *M* TMAC stabilizes A:T base pairs such that they melt at the same temperature as G:C base pairs. This has the effect of making the melting temperature of any hybrid strictly a function of the length of the hybridized region. A collateral benefit is that TMAC sharpens the melting profile of DNA duplexes, so that hybrids that melt over a 5–10°C range in the presence of Na+ melt within 1–1.5°C in TMAC. Using the TMAC method, it is possible to distinguish between hybrids differing in length by as few as 1 bp ***(6)***. We have used the TMAC method to identify novel nuclear hormone receptors ***(7–9),*** homeobox genes ***(10–13)***, TGF-β family members ***(14)***, and P450 family members ***(15)***.

Previously, a degenerate oligonucleotide (TGY GAR GGN TGY AAR GGN TTC TT) was used to identify then novel members of the steroid receptor superfamily ***(7)***. This DNA sequence corresponds to the highly conserved amino acid sequence, CEGCKGFF, found in the P-box ***(16)*** of the DNA-binding domain of many nuclear receptors. It is straightforward to use a similar approach to identify vertebrate homologs of the many recently identified *C. elegans* ORFs. **Figure 1** shows a representative selection of the different P-box sequences from *C. elegans* thought to encode nuclear hormone receptors. Although there are only 25 P-box sequences, these represent more than 200 receptor DNA-binding domains. Because *C. elegans* does contain receptors that harbor the CEGCKGFF P-box, it is not unreasonable to suppose that

```
CAACAAFF
CDGCKGFF        CDACKMFF
CEGCKGFF        CESCKAFF        CESCKGFF
CFGCKGFF
CGACAAFF
CHGCKAFF
CKACAAFF        CKGCKTFF
CLACAAFF        CLGCKTFF
CNGCKTFF        CNGCKGFF        CNACKMFF
CRACTAFF        CRACAAFF        CRGCNAFF
CSACGSFL        CSACSSFF
CTACASFF
CVGCKTFF
CYACKMFF        CYGCKGFF


C   E   G   C   K   G   F   F
TGY GAR GGN TGY AAR GGN TTC TT
```

Fig. 1. P-box sequences for known and putative *C. elegans* nuclear receptors. The oligonucleotide we have successfully used and the corresponding P-box sequence is shown at the bottom.

vertebrate homologs will exist for at least a subset of the different identifiable *C. elegans* P-box sequences uncovered by the genome project. The use of P-box oligonucleotides to screen allows one to identify the particular receptor cloned in a single sequencing reaction.

2. Materials

1. 5.0 *M* TMAC stock solution: TMAC is hygroscopic; therefore, one must prepare a stock solution at a nominal concentration of 5 *M*, then precisely quantitate it with a refractometer (*see* **Note 1**). Calculate the molarity (*M*) from the following formula:

   $$M = (\text{refractive index} - 1.331)/0.018$$ (*see* **Note 2**)

2. TMAC washing buffer: 3.0 *M* TMAC, 0.05 *M* Tris-HCl, pH 8.0, 0.2 m*M* ethylenediamine tetraacetic acid (EDTA).
3. Standard oligonucleotide hybridization buffer (*see* **Note 3**): 6.6 × NET (final 1 *M* NaCl, 0.1 *M* Tris-HCl, 6 m*M* EDTA), 5 × Denhardt's solution, 0.05% sodium pyrophosphate (NaPPi), 0.1% sodium dodecyl sulfate, 0.1 mg/mL, yeast RNA, 125 U/mL heparin.
4. 20X NET: 3 *M* NaCl, 0.3 *M* Tris-HCl, pH 8.3, 18 m*M* EDTA.
5. 6X Standard sodium citrate (SSC), 0.05% NaPPi.

3. Methods

3.1. Designing an Oligonucleotide

3.1.1. Considerations

1. It is useful to also use the screening oligonucleotide for identifying the recombinant clones by sequencing; therefore, try to design the oligo so that a sequence diagnostic for the family is read when it is used as a sequencing primer. This makes the classification of positive cDNAs and the identification of false positives rapid.
2. Since chemically synthesized oligonucleotides are built from the 3' to 5' ends, the 3' end of the oligonucleotide must be unique, or else you will have to make several different oligonucleotides.
3. The degeneracy in the 3'-most 11 nucleotides (nt) makes a difference when using the screening oligonucleotide as a sequencing primer. For best results, try to keep this below 16-fold. To sequence 1 pmol of template (optimal amount), use a molar excess of primer approximately equal to the degeneracy in the 3'-most 11 nt.

3.1.2. Rules of Thumb

1. Use oligonucleotides 20–30 nt in length. The author prefers 23-mers for most screenings.
2. Avoid sequences containing Ser. If this is not possible, make two separate oligonucleotide pools, one with the AGY codons, and the other with the TCN codons.
3. Avoid codon usage tables. Make the oligonucleotides a completely degenerate version of the amino acid sequence. Do not make combination guessmers and degenerate probes (*see* **Note 4**).
4. Try not to make the oligo self-complementary, if that can be avoided.
5. Do not purify degenerate oligonucleotides by ion-exchange chromatography. Pharmacia and others recommend purification on ion exchange resins under alkaline conditions, but, in the author's experience, this fractionates oligonucleotides by sequence and biases the composition of individual fractions.
6. Synthesize degenerate oligonucleotides at the 1 μ*M* scale. Most instruments use the largest amount of excess reagents at this scale and this translates to decreased bias in the resulting oligonucleotide pool.
   a. Have the supplier purify the oligo by polyacrylamide gel electrophoresis (PAGE) or reverse phase (RP) cartridge. PAGE is superior because RP cartridge purification does not remove any trityl-on failure sequences that may be present. This could result in a mixed size population (*see* **Note 5**).
   b. Alternatively, purify the (detritylated) oligonucleotide on a 15% polyacrylamide-8 *M* urea gel. Second choice (for a trityl-on) oligonucleotide would be to purify it on a RP cartridge, such as NENsorb-Prep.

3.1.3. Calculating the Number of Expected False Positives

1. Under the best circumstances, one will still expect to find some false positives that result from random matches between the oligonucleotide and the target DNA sequences.

2. The *a priori* statistical probability of finding a matching sequence in any random DNA sequence is a function of the total number of nucleotides to be screened, size of the oligonucleotide, number of contiguous matches required, and size of the oligonucleotide pool.

$$N = C(2)(n - h + 1)p/4^{h}$$

   where N = the number of expected random matches/haploid genome, C = the genome size (or the complexity of the library * the average size), n = the length of the probe, h = the number of matches required, p = the total number of different oligonucleotides in the pool.
   a. For 10^6 cDNA clones, of average length 2 kb, screened with a mixture of 512 different 23-mers under two mismatch conditions, we get the following: $N = (2000)(10^6)(2)(23 - 21 + 1)(512)/4^{21}$, which is 1.4 expected random matches.
   b. An important factor here is the degeneracy of the probe (*see* **Note 6**).
   c. In the above calculation, the value for C is the total number of independent base pairs screened.
   d. If the cDNA library used were amplified, then one would use the number of independent clones or the number screened, whichever is smallest, for C. For a genomic Southern, use the size of the genome. For a genomic library, divide the genome size by the size of the average insert.
3. Unfortunately, DNA sequences are not random; therefore, one must also search the oligonucleotide sequence against the DNA database to ensure that it does not accidentally hybridize to repetitive sequences or other sequences that may interfere with the screening.
   a. Sometimes this initial screening can help to identify unknown members of the gene family under investigation ***(17)***.
   b. The FINDPATTERNS program of UWGCG works well for this purpose.
   c. Since DNA has two strands, one must also search with the complement of the screening oligonucleotide.

3.2. Labeling the Probe

1. It is important to use enough probe and to make it hot enough.
2. A standard, high-density library screen (e.g., duplicate filters from 10 150-mm plates with 100,000 plaques each) would require ~200 pmol oligonucleotide. For a 23-mer, this is 760 ng (7.6 ng/pmol).
3. In subsequent purifications, 200 pmol oligonucleotide is adequate for only 24 filters or so (100 mm), because, depending on the degeneracy of the probe, one might well not be in probe excess with the higher numbers of phage present during plaque purification. To convince yourself that this is true, do the following calculations.
   a. Assume 10^7 phage/plaque (conservative for λZAP or λgt10), 50 positive plaques/plate average, 24 filters (duplicates from 12 plates), and 200 pmol 1000-fold degenerate oligo.
   b. Calculating molecules of target: $(10^7)*(50)*(24) = 1.2 \times 10^{10}$ targets.

c. Calculating molecules of probe: 200 pmol of oligo = (6.02×10^{23} molecules/mol * 200×10^{-12} mol) = 1.2×10^{14} molecules of probe, divided by the degeneracy (1024-fold) = 1.1×10^{11} of each probe species, assuming 100% labeling. In practice, 100% labeling is not possible, moreover, there will be sequences to which the probe hybridizes only moderately, thus diluting out the available probe for true positives.

4. Be sure to make the probe hot enough.
   a. Use severalfold molar excess of γ^{32}P-adenosine triphosphate (6000 Ci/m*M*, or greater) whenever possible.
   b. Pure γ^{32}P-adenosine triphosphate (e.g., NEG-002Z, New England Nuclear) works best but is expensive when labeling large amounts of probe. One can substitute a crude preparation (e.g., NEG-035C) for this purpose; however, the probes are not as completely labeled. This is a reasonable trade-off considering that it is 5–10-fold less expensive (*see* **Note 7**).
   c. After the labeling reaction is completed, remove the unincorporated label by two consecutive spun columns using Sephadex G-25 or equivalent (*see* **Note 8**).

3.3. Plaque Lifts

1. Plating the library:
   a. For first-round screens, plate the library to obtain 50–100 K plaques/150-mm round plate, or 250,000–500,000 per 22 × 22-cm bioassay dish.
   b. Lift duplicate filters (3 min first lift and 6 min second lift) and do not even consider purifying signals that do not duplicate (*see* **Note 9**).
   c. Process a convenient number of plates at a time, e.g., 6–12, depending on how facile you are. Spread the filters out on large sheets of filter paper until all lifts have been finished and allow them to dry at room temperature.
2. For first-round screens, place the filters plaque-side-up on blotter paper saturated with 0.5 *M* NaOH–1.5 *M* NaCl for 3 min, then on 0.5 *M* Tris-HCl, pH 7.5–1.5 *M* NaCl for 3 min, then on 2X SSC for 3 min (*see* **Note 10**).
3. When processing of each filter or set of filters is completed, transfer to sheets of dry filter paper, and allow to air-dry. Bake nitrocellulose or nylon filters at 80°C for 30 min ***(18)***. Nylon filters may be UV crosslinked, but this does not increase signal strength.
4. Wash the dried filters for 15 min in 0.05 *M* NaOH with shaking. This step removes debris from the filters, enhances the signal, and reduces the background considerably.
5. Rinse the filters in five, 3-min changes of dH_2O to ensure removal of NaOH. Check the final wash with pH paper to ensure that NaOH has been removed.

3.4. Hybridization

1. Prehybridize overnight in hybridization buffer at 42–46°C (*see* **Note 11**).
2. Calculating the hybridization temperature:
   a. Optimally, one should hybridize at T_m – 5°C for perfectly matched probes. This is impossible for mixed-probe populations; hence, calculate the maxi-

mum number of possible A and T residues possible for the probe, then estimate the minimum T_m assuming $T_m = 4°C *(G + C) + 2°C *(A + T)$.
   b. Hybridize at ~10–15°C below this T_m (*see* **Note 12**), 46°C works well in practice for 23-mers.
3. Overnight hybridization is sufficient. Longer times give higher background.
4. The volume is not critical since the probe cannot self-anneal. Use about 10–15 mL/bag.
5. Incubation with agitation is not necessary, but not harmful if a shaker or hybridization oven is available.

3.5. Washing

1. This is the most critical step. The most important factor in a successful screen is careful and skillful washing. Do not take shortcuts or deviate from the protocol.
2. Calculate the correct washing temperature:
   a. Obtain the T_m for a specific-length oligonucleotide from the figure in **ref. *6***.
   b. Assume that T_m is reduced by 1°C for each % mismatch.
   c. Wash at T_m – 5°C – (mismatch reduction).
   d. For a 23-mer at 1 mismatch, this is 65°C – 5 – 4 = 56°C (*see* **Note 13**).
3. After hybridization, remove the filters to a container of 500 mL–1 L 6X SSC, 0.05% NaPPi. Rinse for 2 min at room temperature to remove unhybridized probe.
4. Remove the SSC, add a fresh aliquot, and incubate at room temperature with shaking for 15 min. Take care that the filters move around freely and do not stick to each other or to the container.
5. Remove the SSC and add a sufficient amount of 3 *M* TMAC wash buffer so that the filters can move freely when agitated. Incubate 15 min at room temperature (*see* **Note 14**).
6. Place the filters in a seal-a-meal bag and leave sufficient area for the filters to move around freely (*see* **Note 15**).
7. Add 200 mL preheated TMAC wash buffer and place the bag with filters into a preheated water bath. Anchor the corners so that the bag does not move, but allows the filters to move freely.
8. Incubate with shaking for 15 min. Cut the corner of the bag, remove the TMAC, add another preheated aliquot, and repeat the washing (*see* **Note 16**).
9. Place the filters back into the larger container and wash with 6X SSC, 0.05% NaPPi to remove TMAC, which smells bad and leaves a sticky residue on the filters.

3.6. Detecting the Positive Signals

1. Place the wet filters between sheets of plastic wrap and fold the edges over to prevent drying.
2. Tape the filters securely to a sheet of used X-ray film or other suitable transparent or translucent support.
3. Place tiny dots of radioactive ink on the tape for orientation purposes.
4. Expose to film for 1–3 d with intensifying screens at –80°C (*see* **Note 17**).
5. Orient the cassette (bottom to top) as sample, screen, film, screen. When using BioMax MS screens and film, one screen is typically sufficient. In this case, the orientation should be sample, screen, film.

3.7. Purifying Positive Signals

1. After developing the film, orient the films to the filters by aligning the spots of radioactive ink with the signals they produce.
2. Transfer the filter labels and the registration marks in the filter to the films by marking with a felt-tip pen.
3. Align the registration marks on the first and second lift and circle signals that duplicate.
4. After finishing all filters, place the film with the signals circled on a light box.
5. Align the holes in the plate with the registration marks on the film.
6. Pick a region surrounding the signal with the blunt end of a Pasteur pipet (or a yellow tip cut to about one-third its original length.
7. Transfer the plug to a 1.5-mL microcentrifuge tube containing 1 mL SM buffer. Allow the phage to diffuse out of the plug for at least 4 h, and preferably overnight.
8. For subsequent rounds of screening, dilute the primary plaque 1000-fold and plate three dilutions (e.g., 1, 5, and 25 μL). Select the plate with an appropriate number of plaques for lifting filters.
   a. For a second-round screen, pick a plate that has about 100–500 plaques and one higher density for each positive.
   b. Pick only one positive signal per second round plate and try to pick one that is separated from surrounding plaques by the widest margin. If this margin is >5 mm all around, the plaque is probably pure. If not, perform another round of screening.
   c. For third round screens, 25–50 plaques/plate is good but do not use a plate with less.

3.8. Sequencing Positive Clones

1. Another advantage of using degenerate oligonucleotides to screen cDNA libraries is that the cDNAs may be directly identified by sequencing them with the screening oligo.
2. In general, best results are obtained when sequencing 1 pmol DNA with a 1–2-fold molar excess of primer.
3. For degenerate primers, a larger molar excess is required. A rule of thumb is to calculate the degeneracy in the 3'-most 11 positions of the primer, then use this molar excess. For optimal results, the amount must be calculated and titrated for each different primer.

4. Notes

1. If you do not have a refractometer, find someone who does. If a refractometer cannot be found, use a brixmeter (e.g., 28–62% sugar, Fisher no. 13-946-60B). Read the TMAC as % sucrose and convert this to refractive index using the table in the CRC handbook entitled “Index of refraction of aqueous solutions of sucrose.” 5 *M* TMAC has a refractive index approximately equal to that of 50% sucrose.

2. The author usually buys a whole case of TMAC from Fisher, dissolve it all at ~5 *M*, pool into a large flask, mix well, filter to remove debris, then measure a small aliquot. Dispense the stock into conveniently sized aliquots. Store in tightly sealed bottles to avoid absorption of H_2O and subsequent changes in concentration.
3. Filter the hybridization buffer through a 0.45-μm filter, preferably composed of the same type of membrane that will be used for hybridization and store at 4°C. This filtration step results in reduced background.
4. The author has never failed to clone the desired sequence using completely degenerate oligonucleotides, but has had bad experiences with probes containing inosine instead of mixed nucleotides. Consequently, these are not recommended.
5. The authors has had excellent success with degenerate oligonucleotides produced by Genosys (www.genosys.com).
6. One can use pool sizes of up to 3000 or so, but the best results are obtained with 1024-fold degenerate (or less) 23-mers. The oligonucleotide the author used for identifying novel orphan receptors was 512-fold degenerate.
7. Use the purified isotope (NEG-002Z) for labeling the probe for high-density, first round screening, then use the crude isotope (NEG-035C) to label the probe for subsequent rounds of purification.
8. One spun column typically gives ~95% removal of the unincorporated label, however, considering the amounts of isotope in use here (1–10 mCi), this leaves too much free label (50–500 μCi) remaining. This can result in high background. The second column reduces this to 2.5–25 μCi, which is acceptable.
9. Ensure proper alignment by poking asymmetric, vertical holes through the filter and into the plate during the first adsorption. After all of the first lifts are complete, place the second filter on each plate, then hold each plate up to the light, and precisely duplicate the hole pattern. Performing duplicate lifts for first- and second-round screens is important to ensure that only true positives are picked; it is optional (but safer) for third-round screens.
10. It is important for subsequent signal strength that high-density filters from first round screens be processed by capillary action. For second- or third-round screens, it is acceptable to process the filters by immersion in containers of the solutions. Incubate for 3 min with enough shaking to keep the filters from sticking to each other.
11. This extended prehybridization reduces background considerably. Prehybridization temperature is not critical. For convenience, one typically uses the same temperature for both hybridization and prehybridization.
12. The author typically hybridizes up to 25 filters/bag and uses 200 pmol of labeled probe/hybridization. Do not exceed this number or low and variable signal will result.
13. For a 23-mer, one mismatch is ~4°C, two mismatches is ~8°C. The author empirically found that 56–58°C is optimal for washing 23-mers. Use the higher temperature for less degenerate probes. Alternatively, test the washing conditions using Southern blots and cloned sequences that should be detected by the probe.

14. This step exchanges sodium ions from the SSC for tetramethyl ammonium ions in the TMAC washing solution, which is important for effective washing.
15. An area of about 25 × 25 cm is adequate.
16. Doing the washing in a sealed bag is *essential* for proper temperature equilibration and control. In principle, use of a hybridization oven for these washing steps should be possible, but, in practice, it does not seem to work very well.
17. The type of film and intensifying screen used makes a very big difference for degenerate oligonucleotide screens (and other applications in which the signal strength is likely to be low). The combination of green-emitting screens and green sensitive film (e.g., Kodak BioMax MS screens and film) gives an approximately eightfold increase in signal over Kodak XAR-5 film (blue sensitive) and standard (blue-emitting) intensifying screens (e.g., Lightning Plus) and 2–3-fold increase in signal over a blue emitting film (e.g., XAR-5) and blue emitting rare-earth screens (e.g., Quanta III). Moreover, the BioMax MS film/screen combination gives sensitivity comparable to using a Phosphorimager.

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III

Steroid Hormone-Binding Assays

10

Use of [^{99m}Tc]Technetium-Labeled Steroids as Probes for Steroid Hormone Receptors

Frank Wüst

1. Introduction

Nature has made extensive use of metals in biological systems. Metals perform a wide variety of essential biological functions, such as oxygen and electron transfer, the development of structural framework, and they represent the reactive centers in catalytic proteins. The investigation of the biological role of metals and their coordination chemistry in a biosystem is the subject of bioinorganic chemistry ***(1,2)***.

The use of coordination chemistry for diagnostic or therapeutic medicine purposes is termed medicinal inorganic chemistry ***(3)***. In diagnostic medicinal inorganic chemistry, the introduction of radionuclides as molecular probes provides vital information on biological processes at a molecular level. This concept of an in vivo biochemistry is realized in modern nuclear medicine, which is the field of medical practice that involves the oral or intravenous administration of radioactive drugs, the radiopharmaceuticals. After administration, the radiopharmaceutical localizes within an organ or target tissue because of its biological or physiologic characteristics ***(4)***. Information on tissue shape, organ function, and physiologic processes are obtained by images, which are generated by the radioactivity distribution within an organ or at a location within the body.

The most commonly used radiometal for such a radioimaging is the γ-emitter, technetium-99m (^{99m}Tc). Its widespread availability from the commercial (^{99}Mo)/^{99m}Tc generator system, its convenient half-life ($t_{1/2}$ = 6 h), and appropriate γ-energy (140 keV), accounts for ^{99m}Tc being the "workhorse" of modern nuclear medicine in over 80% of all routine diagnostic nuclear medicine procedures. ^{99m}Tc-containing radiopharmaceuticals include perfusion agents for the

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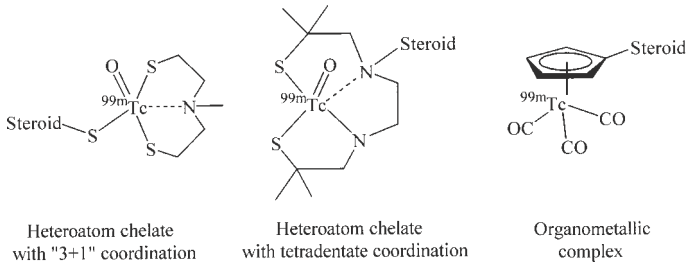


Scheme 1. Stabilization of ^{99m}Tc by chelation or organometallic species.

heart (^{99m}Tc-MIBI [Cardiolite®]) and the brain (^{99m}Tc-ECD [Neurolite®]) and ^{99m}Tc-hexamethyl-propyleneamine oxime (Ceretech), as well as an agent for renal function (^{99m}Tc-MAG_3) ***(5)***.

In the current forefront in radiopharmaceutical chemistry, efforts are devoted to the synthesis of ^{99m}Tc-labeled small molecules intended to be ligands for specific hormone, neurotransmitter, or drug receptors, as well as specific, high-affinity transport systems and enzymes ***(5–9)***. Because these receptors are known to be involved in the regulation of vital body functions, effective imaging agents can be used in the diagnosis or staging of a variety of disease states, in which such receptors are functioning or distributed in an abnormal fashion.

However, despite its beneficial physical properties and availability, the use of ^{99m}Tc in probing and imaging specific biological targets, such as steroid hormone receptors poses a major challenge. The nonphysiological metal, ^{99m}Tc, must be adapted to the biological environmental by means of coordination chemistry. As a d-block transition metal, ^{99m}Tc must be incorporated into small-molecule receptor ligands, such as steroids, by some chelation system, which may involve multiple heteroatom coordination ***(9–19)***, or by the formation of stable organometallic species ***(17–20)*** (**Scheme 1**).

In the case of specific agents, such as steroid hormones, the used chelation systems or organometallic species are bulky and have a mass comparable to that of the receptor ligand itself. Consequently, the conjugation with such a metal complex results in a molecular weight increase at least doubling the size of the receptor ligand itself, which may diminish receptor affinity through steric interference, and alters physicochemical properties significantly.

However, recent promising results in the design of ^{99m}Tc-labeled steroids show that several ^{99m}Tc-containing estrogen and progestin conjugates retain a remarkably high binding for the corresponding estrogen receptor (ER) and progesterone receptor, respectively ***(10–12,16–19)***.

The present contribution on steroid receptor methods and protocols wants to describe the procedures of the most common coordination chemistry tools to incorporate ^{99m}Tc into steroid hormones to probe steroid hormone receptors.

2. Materials

2.1. Chemical Syntheses

1,1'-*bis*(methoxycarbonyl)ferrocene, for the synthesis of the ^{99m}Tc-containing organometallic complex, was prepared as previously described ***(21)***. The tridentate ligand, *bis*(2-mercapto-ethyl)methylamine, for the synthesis of the ^{99m}Tc complex with "3+1"-coordination, was prepared according to a literature procedure ***(22)***.

The functionalized steroid precursors were prepared according to multistep organic syntheses protocols as previously described.

1. 7α-(6-Mercaptohex-1-yl)estra-1,3,5(10)-triene-3,17β-diol and 7α-(6-aminohex-1-yl)-*bis*(*tert.*-butyldimethylsilanyloxy)estra-1,3,5(10)-triene were prepared, starting from commercially available 3,17β-estradiol ***(18)***.
2. 11β-[α-[N-[2-[N-(2'-methyl-2'-mercaptopropyl)amino]ethyl]-*N*-(2'-methyl-2'-mercapto-propyl)amino-*p*-tolyl]-17α-propynyl-17β-hydroxy-4,9-estra-dien-3-one was prepared starting from commercially available 3-methoxyestradiol ***(10)***.

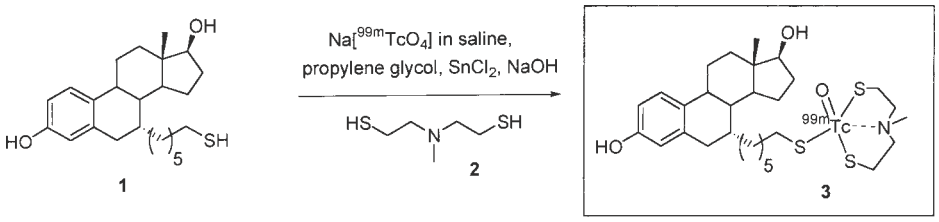
All other chemical reagents and solvents were purchased from commercial sources (Aldrich, Fluka, Acros, Alfa Aesar) and used as received.

2.2. Mo-99/Tc-99m Generator

The ^{99}Mo/^{99m}Tc generator provides a convenient source of Na[$^{99m}TcO_4$] as pyrogen-free isotonic solution for immediate oral or intravenous administration, or for the preparation of labeled radiopharmaceuticals. The ^{99}Mo/^{99m}Tc generator is commercially available (Mallinckrodt, DuPont/Pharma, Amersham), and the Na[$^{99m}TcO_4$] can be obtained by elution of the generator with saline using an evacuated elution vial (provided with the generator). The generator is generally eluted on a 24-h schedule. Each generator allows up to 10 elutions. The eluted radioactivity depends on the age of the generator and the elution schedule.

2.3. Special Remarks for the Work with Radioactivity

The user should wear protective clothing (lab coat, shoe covers, gloves, and safety glasses) and use appropriate shielding at all times when handling radioactivity. Whenever possible, operations with radioactive materials should be conducted in a fumehood. In order to keep exposure to radiation as low as possible, the user should remember the three "golden rules:"



Scheme 2. Synthesis of [3-(*N*-methyl)azapentane-1,5-dithiolato][7α-6-mercaptohex-1-yl) estra-1,3,5(10)-triene-3,17β-diol]oxo-[^{99m}Tc](V) ***(3)***.

1. Time: Plan the experiment carefully in advance and minimize time to avoid unnecessary long exposure times when handling radioactivity.
2. Distance: Avoid touching radioactivity-containing glassware and other equipment directly. Work with tools (tweezers, and so on) to guarantee the maximum distance to the radioactive sample (the dose is reduced by the square of the distance).
3. Shielding: Use appropriate shielding (lead) for all manipulations (lead container for reaction vials, working behind lead bricks, and so on).

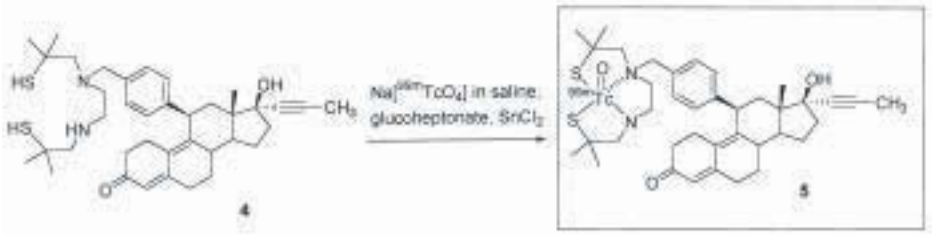
3. Methods

The following subheadings describe in detail the individual steps necessary to incorporate ^{99m}Tc into steroids employing several coordination chemistry tools according to two heteroatom chelation approaches and one organometallic approach.

3.1. ^{99m}Tc-Labeled Steroids Containing Heteroatom Chelation with "3+1" Coordination: Synthesis of [3-(N-methyl) azapentane-1,5-dithiolato][7α-6-Mercaptohex-1-yl) Estra-1,3,5(10)-triene-3,17β-diol]oxo-[^{99m}Tc](V) (3)

The principle of the heteroatom chelation with 3+1 coordination consists of the saturation of three coordination sites of the oxotechnetium(V) core by a small tridentate SNMeS ligand and filling the remaining fourth position with a monodentate coligand bearing the functionalized biomolecule (steroid) ***(23)***. The preparation of the estrogen receptor affine ^{99m}Tc-complex was carried out by a ligand-exchange reaction starting from ^{99m}Tc-propylene glycolate as the labeling precursor (**Scheme 2**).

1. Add 0.45 mg monodentate thiol ligand 1 in CH_3CN (100 μL), 0.05 mg of tridentate ligand 2 (10 μL, 5 mg stock solution in 1 mL CH_3CN) and 0.1 *N* NaOH (50 μL) to 1 mL of Na[$^{99m}TcO_4$] in saline (35 mCi of generator eluate) and 1 mL propylene glycol in a 10-mL reaction vial, which is inserted into a small lead container.



Scheme 3. Synthesis of 11β-N-[α-Oxo(*N,N'-bis*(2'-methyl-2'-mercaptopropyl) ethylenediaminato)-[^{99m}Tc](V)-p-toluyl]-17α-propynyl-17β-hydroxy-4,9-estra-dien-3-one ***(5)***.

2. Add 10 μL Sn(I-I)Cl_2 solution (1–2 mg Sn(II)$Cl_2 \cdot 2H_2O$ in 5 mL 0.1 *N* HCl) to the solution.
3. Close the vial and incubate the mixture with the lead container at 50°C for 20 min by means of a water bath.
4. Transfer the mixture into a 10-mL round bottom flask and evaporate the solvent under reduced pressure with a rotary evaporator.
5. Take up the residue (0.5 mL) into a syringe and inject it onto a semipreparative Hypersil (RP-18) column (Isocratic elution: MeOH/0.01 phosphate buffer, pH 7.4 (80/20); flow rate 3 mL/min.
6. Complex 3 has a retention time of 7.4 min.
7. Collect the fractions containing the radioactivity into a 25-mL round bottom flask and remove the solvent by vacuum evaporation.
8. Complex 3 has a radiochemical purity of >96%; the overall decay-corrected yield is 95%.
9. Add propylene glycol (100 μL) to the flask to assist solubility of complex 3 prior the addition of 10% ethanolic saline for further studies (receptor-binding studies, biodistribution, and so on).

3.2. ^{99m}Tc-Labeled Steroids Containing Heteroatom Chelation with Tetradentate Coordination: Synthesis of 11β-N-[α-Oxo (N,N'-bis(2'-methyl-2'-mercaptopropyl)-ethylenediaminato)-[^{99m}Tc](V)-p-toluyl]-17α-propynyl-17β-hydroxy-4,9-estra-dien-3-one (5)

The synthesis of the ^{99m}Tc heteroatom chelate with tetradentate coordination with a 11β-functionalized progestin was accomplished through a ligand-exchange reaction, using ^{99m}Tc-glucoheptonate as the labeling precursor. Only the syn diastereomeric pair of the possible stereoisomers is formed (**Scheme 3**).

1. Add 2 mL of Na[$^{99m}TcO_4$] in saline (100 mCi generator eluate) to a Glucosan kit (200 mg glucoheptonate, 0.06 mg Sn(II)Cl_2), swirl, and allow to stand for 15 min at room temperature (RT) in a small lead container.

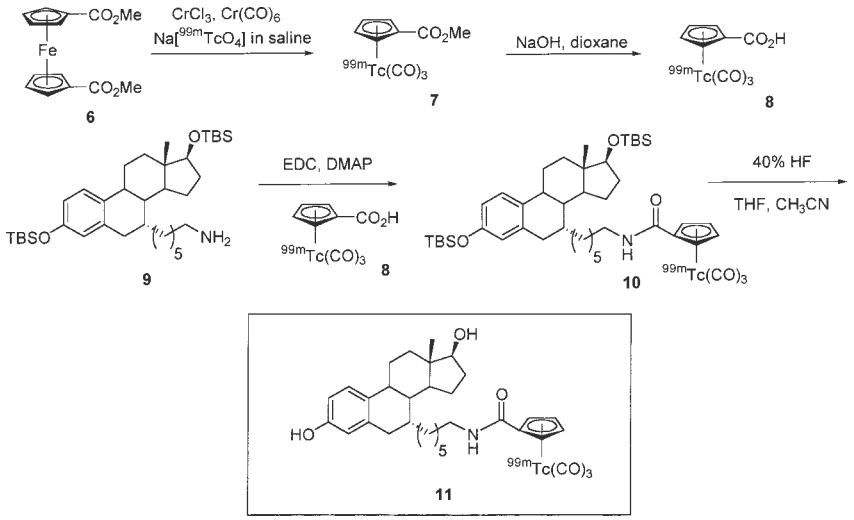
2. Add a 100-μL aliquot of the formed ^{99m}Tc-gluconate solution (4.5 mCi) with a 1-mL disposable syringe to a solution of steroid 4 (0.5 mg) in 100 μL MeOH in a 3-mL sample vial with a stir bar.
3. Stir the mixture for 15 min at room temperature.
4. Dilute the mixture with 0.5 mL saline and transfer it into a 10-mL round bottom flask.
5. Rinse the sample vial with CH_2Cl_2 (3 × 1 mL) and use the same CH_2Cl_2 to extract the saline solution (3 × 1 mL CH_2Cl_2) by means of a Pasteur pipet (the bottom layer [CH_2Cl_2] is removed with a Pasteur pipet after each extraction).
6. Dry the combined organic layers by passage through a Pasteur pipet filled with $MgSO_4$ and evaporate the solvent with a stream of nitrogen (N_2).
7. Redissolve the residue in 1 mL 50% CH_2Cl_2/*n*-hexane and inject it onto a preparative silica column (Whatman Partisil M-9, 0.9 × 50 cm; mobile phase: 35% (1/20 iPrOH/ CH_2Cl_2)/65% *n*-hexane; flow rate: 5 mL/min).
8. Combine the fractions containing the radioactivity in a 25-mL round bottom flask and remove the organic solvents by vacuum evaporation.
9. Complex 5 has a retention time of 14 min and the overall decay-corrected yield is 43%.
10. Add propylene glycol (100 μL) to the flask to assist solubility of complex 5 prior the addition of 10% ethanolic saline for further studies (receptor-binding studies, biodistribution, and so on).

3.3. ^{99m}Tc-Labeled Steroids Containing an Organometallic Moiety: Synthesis of Tri-carbonyl-{[6-[estra-1,3,5(10)-triene-3,17β-diol-7α-yl]-hexylamido]cyclopentadienyl}-[^{99m}Tc]technetium (I) (11)

The synthesis of an organometallic ^{99m}Tc–estradiol complex begins with the formation of cyclopentadienyltricarbonyltechnetium-99m carboxylic acid as the labeling precursor via a double ligand transfer reaction, starting from 1,1'-*bis*(methoxycarbonyl)ferrocene 6 ***(24)***. The incorporation of the organometallic ^{99m}Tc-moiety into the functionalized steroid 9 was accomplished by means of a coupling reaction followed by the removal of the silylether-protecting groups (**Scheme 4**).

3.4. Cautionary Note

Pressure tubes were placed within a solid aluminum block containing holes drilled deep enough to admit the tubes to about three-fourths of their height and wide enough to allow room for the addition of some mineral oil to ensure good thermal contact. The tubes and aluminium base were covered with a matching hollow aluminium screw cap, equipped with a small hole to hold a thermometer. This device minimizes the potential danger of explosions during heating and enables the monitoring of the reaction temperature.



Scheme 4. Synthesis of tricarbonyl{[6-[estra-1,3,5(10)-triene-3,17β-diol-7α-yl]-hexylamido]cyclopentadienyl}-[^{99m}Tc] (I) ***(11)***.

1. Transfer an aqueous solution of [Na^{99m}TcO$_4$] in saline (80 mCi of generator eluate) to a 4 mL thick-walled pressure tube (Ace glass) equipped with an egg-shaped (1 cm) stir bar.
2. Remove the water azeotropically under a steady stream of nitrogen at 50°C with periodic addition of acetonitrile.
3. Add 1,1'-*bis*(methoxycarbonyl)ferrocene 6 (10 mg, 33.1 mmol), $CrCl_3$ (4 mg, 25.3 mmol), $Cr(CO)_6$ (14 mg, 63.6 mmol), and MeOH (0.5 mL).
4. Seal the pressure tube with a Teflon screw cap equipped with an O-ring.
5. The reaction is heated quickly to 185°C with a heat gun and the temperature is maintained with a hot plate for an additional 30 min.
6. Remove the pressure tube carefully from the heating block: First cool to room temperature in a water bath, then to 0°C in an ice bath, and finally to –78°C in a dry ice-isopropanol bath.
7. Open the pressure tube carefully and remove the green solution with a 2.5-mL polypropylene syringe and place the solution into a disposable scintillation vial.
8. Wash the tube several times with CH_2Cl_2 and add the washings to the scintillation vial.
9. Evaporate the solvent in the scintillation vial under a stream of N_2 at 45°C.
10. Redissolve the green residue in a minimum amount of CH_2Cl_2 and load it onto a 5-mL Pasteur pipet containing 8 cm silica.
11. Elute the column with CH_2Cl_2 (6 mL) until the orange band is just to be eluted.
12. The first 6 mL CH_2Cl_2 eluted from the column contain substantially pure tricarbonyl(methoxycarbonylcyclopentadienyl)technetium-99m 7.

13. Concentrate the CH_2Cl_2 eluate under a stream of N_2 and, using a 50°C oil bath, transfer the residue (approx 1 mL) with a Pasteur pipet to a 3-mL sample vial containing a stir bar.
14. Evaporate the CH_2Cl_2 to dryness under a stream of N_2 at 50°C.
15. Redissolve the residue in dioxane (100 μL) and 2 *M* NaOH (300 μL) and stir the solution vigorously for 10 min.
16. Add concentrated HCl (60 μL) and take the solution up in a 2.5-mL polypropylene syringe.
17. Load the mixture onto an activated C-18 Light SepPak (activation: first 6 mL EtOH, followed by 6 mL H_2O).
18. Remove the plunger of the syringe and elute the Sep-Pak with H_2O (5 mL).
19. Elute the carboxylic acid 8 with EtOH (700 μL), which was collected in 100-μL fractions.
20. Discard the first fraction (100 μL). The others are combined in a 3-mL sample vial containing 1 mg of the amine 9 and a stir bar.
21. Remove the solvent under a gentle stream of N_2 at 50°C.
22. Redissolve the residue in CH_2Cl_2 (300 μL) and add 1-(3-dimethylaminopropyl)-3-ethylamine carbodiimide · HCl (5 mg) and some crystals of dimethylamino-pyridine.
23. The vial is fitted with a small rubber septum that has a needle inserted through the top and the solution is stirred for 10 min at room temperature.
24. Take up the solution into a 1-mL disposable syringe and the mixture is loaded onto a normal phase silica Light Sep-Pak.
25. Elute the Sep-Pak with ethyl acetate (EtOAc) (500 μL) into a 3-mL sample vial with a stir bar and evaporate the solvent under N_2 at 50°C. The eluate contains amide 10.
26. Add tetrahydrofuran hydrofluoric acid (THF) (160 μL), CH_3CN (120 μL), and 40% HF (70 μL) to the 3-mL vial containing the amide 10.
27. The sample vial is fitted with a small rubber septum and a needle.
28. Heat the solution to 60°C for 15 min, then neutralize with saturated $NaHCO_3$ (100 μL) solution, followed by solid $NaHCO_3$ until bubbling stops.
29. Concentrate the solvent under N_2 at 60°C until a small amount of liquid remains.
30. Add EtOAc (600 μL) and the suspension is taken up into a 1-mL disposable syringe.
31. The syringe is fitted with a Xpertek 13-mm nylon syringe filter, tightly attached and secured to the syringe with parafilm.
32 Pass the suspension through the filter directly into a 2.5 mL polypropylene syringe fitted with a normal-phase silica Light Sep-Pak.
33. Pass the solution through the Sep-Pak.
34. Rinse the reaction vial three times with an additional 300 μL of EtOAc and repeat the sequence with the same filter and Sep-Pak.
35. After evaporation of EtOAc under a stream of N_2 at 50°C, redissolve the residue containing complex 11 in 2.5 mL 70% EtOAc/*n*-hexane. Inject onto a Whatman 46-cm Partisil semipreparative column (mobile phase: 70% EtOAc/*n*-hexane, flow rate: 7 mL/min.

36. Complex 11 has a retention time of 7.70 min and has a radiochemical purity of 96%; the overall decay-corrected yield is 17%.
37. Collect the fraction containing the radioactivity in a round bottom flask.
38. After the evaporation of the solvents, 100 µL propylene glycol is added to assist solubility prior the addition of 10% ethanolic saline for further studies.

4. Notes

The prepared ^{99m}Tc–steroid complexes 3, 5, and 11 exhibited high binding affinity for the corresponding SR (estrogen receptor complex 3 and 11; progesterone receptor complex 5), efficient in vitro stabilities (incubation in serum), and their in vivo biodistribution profile was evaluated in immature female mice (complex 3) and rats (complex 11 and 5), respectively ***(11,12,19)***. These promising results show the possibility of labeling highly specific small molecules, such as steroids, with bulky ^{99m}Tc-chelates, while retaining binding to the corresponding steroid receptor. However, none of the complexes proved to be useful in vivo as receptor-directed agents for diagnostic imaging, presumably because of the high nonspecific binding caused by the increased lipophilicities of the complexes ***(11,12,19)***. Further efforts are underway to use modified, less lipophilic systems as receptor-directed agents for the in vivo imaging of steroid hormone receptors by means of the readily available radionuclide ^{99m}Tc.

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11

Steroid Hormone Metabolites and Hormone Binding Assays

Rosemary Bland and Martin Hewison

1. Introduction

There are two key elements that regulate steroid hormone action, as summarized in **Fig. 1**. First, steroid hormones regulate gene transcription by binding to specific intracellular receptors. The receptors are structurally homologous members of the steroid/thyroid hormone receptor superfamily, and they act as ligand-dependent transcription factors to either activate or repress target gene expression ***(1,2)***. They regulate gene transcription by binding to hormone response element DNA sequences, either as homodimers or heterodimers, with the retinoid X receptor functioning as a common heterodimeric partner ***(3,4,5)***. Therefore, the number of receptors and their binding affinity are important determinants of steroid action, as is the availability of ligand.

Previous studies have focused on circulating levels of steroid. It had been assumed that the lipophilic steroids enter the cell by simple diffusion. However, it is becoming increasingly apparent that membrane-bound molecules may be required for the endocytosis of steroids into the cell ***(6)***. A more well-characterized and crucial factor in determining the action of some steroid hormones in peripheral tissues is the local synthesis and metabolism of the steroid hormone itself. This prereceptor mechanism, which has been term "intracrinology" ***(7)***, is mediated through a series of enzymes expressed in a tissue-specific manner. Thus, for any given peripheral tissue, steroid hormone action may be a reflection of both hormone metabolism and receptor binding. This chapter considers both of these aspects and describes protocols for the analysis of steroid hormone metabolizing enzymes and receptor-binding assays.

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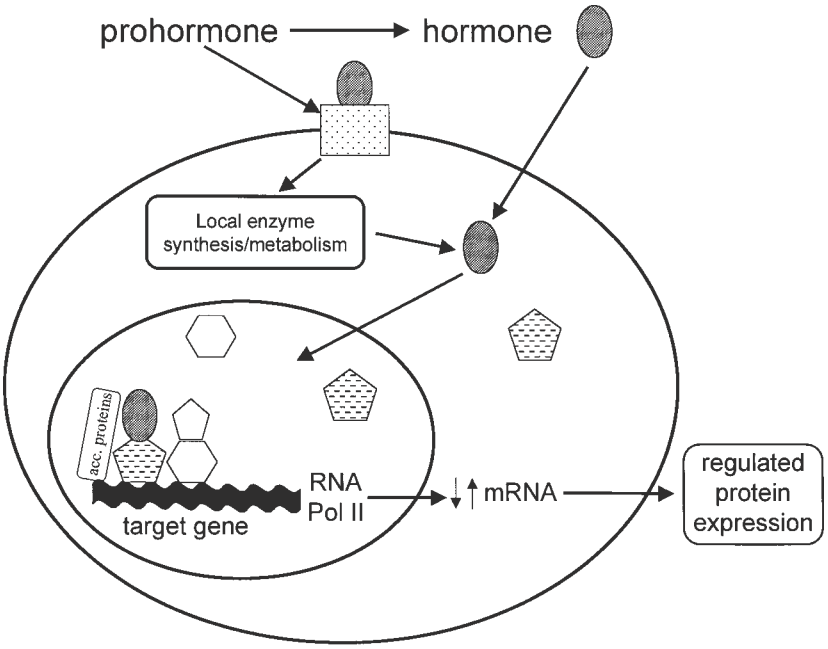

Fig. 1. The main steps required for steroid hormone gene activation. Circulating hormone is converted into the biologically active form, which then enters the target cell. This may occur by passive diffusion or endocytosis. The steroid then either travels to the nucleus to bind to its receptor, or if the receptor is cytosolic, the ligand-receptor complex translocates to the nucleus. Once in the nucleus, the steroid-receptor complexes bind to target gene DNA either as homodimers or as heterodimers, and in conjunction with a variety of accessory proteins, regulate target gene transcription.

2. Materials

2.1. HPLC Analysis of Vitamin D Metabolites

1. High-performance liquid chromatography (HPLC) grade hexane, methanol, isopropanol, acetonitrile, and distilled water.
2. HPLC system equipped with a liquid chromatography spectrophotometer linked to a chart recorder and a fraction collector. If available, a direct radioactivity flow detector is useful, but not essential.
3. Zorbax-sil column (4.6 × 250 mm).
4. Tritiated substrate: (25-Hydroxy[26,27-methyl-^{3}H]cholecalciferol, 180 Ci/mmol (^{3}H-25(OH)D_3) (Amersham Life Sciences, UK).
5. Standard vitamin D metabolites: 25(OH)D_3, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ (25,26$(OH)_2D_3$ and 1,24,25$(OH)_3D_3$ optional standards).
6. Disposable glass tubes, 12 × 100 mm, and a size suitable for use in the fraction collector.

7. Scintillation vials and fluid.
8. Sample concentrator attached to nitrogen or a suitable vacuum drying system.
9. Serum-free cell culture medium.

2.2. Thin-Layer Chromatography Analysis of Androstenedione Metabolism

1. HPLC-grade chloroform and ethylacetate.
2. Serum-free cell culture medium.
3. Tritiated substrate: [1,2,6,7-^{3}H]Androst-4-ene-3,17-dione (^{3}H-androstenedione), 100 Ci/mmol, Amersham).
4. Standard metabolites: [2,4,6,7-^{3}H]estrone (^{3}H-estrone), 100 Ci/mmol (Amersham), 2,4,6,7-^{3}H]estradiol (^{3}H-estradiol), 100 Ci/mmol (Amersham), and 2,4,6,7-^{3}H]testosterone (^{3}H-testosterone), 100 Ci/mmol (Amersham).
5. Disposable glass tubes, 12 × 100 mm.
6. Sample concentrator attached to air and able to heat to 56°C or a suitable vacuum-drying system.
7. Silica thin-layer chromatography (TLC) plates and TLC tank.
8. TLC plate scanner.

2.3. Steroid Hormone Receptor-Binding Assay

1. Ice cold phosphate-buffered saline (PBS), pH 7.4.
2. Ice-cold ethanol.
3. Serum-free cell culture medium.
4. Trypsin solution to remove cells from cell culture flasks.
5. Lysis buffer (store at 4°C): 0.25 *M* sucrose, 0.2 *M* Tris-HCl, pH 7.4, 0.5% Triton X-100.
6. Stock of unlabeled dexamethasone 1×10^{-2} *M* (store at –20°C), diluted in ethanol to give the working concentrations described below.
7. ^{3}H-dexamethasone 1×10^{-6} *M* in ethanol ([1,2,4,6,7-^{3}H]dexamethasone, 100 Ci/mmol, Amersham), diluted in ethanol to give the working concentrations described in **Subheading 3.2.2.1.**
8. RU752 and RU486 (optional) (Hoechst Marion Roussel, Kansas City, Mo).
9. Disposable glass tubes (12 × 75 mm).
10. Scintillation vials and fluid.
11. 37°C Water bath.

3. Methods

3.1. Analysis of Enzyme Activity

Enzyme activity is usually determined by measuring the rate of conversion of substrate to product. There are a number of approaches that can be used to determine the metabolism of steroids: including techniques such as combined gas chromatography/mass spectroscopy *(8)* or radioimmunoassay or enzyme linked immunosorbant assay. However, this chapter concentrates on HPLC and TLC analysis.

3.1.1 Background to Analysis by HPLC or TLC

HPLC and TLC allow the separation of a mixture of closely related substances. Both techniques are based on similar principles; i.e., the flow of liquid (the liquid phase) through a region of immobilized substance (the solid phase) leads to the differential migration of components. In HPLC, the solid phase consists of small particles packed in a column. The small particle size (usually <10 μ*M*) allows for the use of high flow rates, while maintaining good sample separation. In TLC, the solid phase is spread as a thin layer on a flat, firm support to produce a plate. In TLC, the solvent migrates up the plate by capillary action. Separation of molecules by HPLC is carried out in a closed system (HPLC column), through which the solvent is pumped. Separation can occur as a result of sieving, ion exchange, adsorption, or differential solubility. In the HPLC and TLC protocols given below, separation occurs by differential solubility. The liquid phase consists of a mixture of components with varying degrees of polarity. In liquid phases that are relatively nonpolar, relatively hydrophobic substances elute more readily than hydrophilic substances. These basic rules usually apply to silica-based HPLC and TLC systems, which are the most commonly used for analysis of steroid hormone metabolism. However, steroids can also be separated by using systems eluted with more polar solvents. Frequently cited examples of this are the carbon-18 (C-18) HPLC columns which are eluted with mixtures of organic solvents (such as methanol) and H_2O. Regarding HPLC, this approach is often referred to as "reverse-phase HPLC," to distinguish it from the straight-phase, nonpolar solvent systems described above (**Fig. 2B**). The extent of migration of a substance is expressed as the R_f in TLC (R_f = distance traveled by substance/distance traveled by solvent front), and the retention time (time taken for the substance to leave the column) in HPLC.

Both HPLC and TLC are well-established techniques. TLC gives good resolution and reproducibility, and is also quick, easy, and relatively inexpensive. HPLC gives high sensitivity, resolution, and reproducibility, but can be more time-consuming and expensive. Although initial setup costs and maintenance requirements are greater for HPLC, it is more likely to be the most appropriate technique for analysis of multiple metabolites. Both HPLC and TLC can be used as preparative techniques, but in HPLC, the separated steroid molecules can be more easily collected for further studies or additional purification. Equipment and expertise available in a laboratory are most likely to determine the choice of separation technique used. In this respect, the rate limiting component in both HPLC and TLC analysis of steroid metabolism will be the detection systems used to analyze and report the separated steroid metabolites. It is possible to use unlabeled steroids as substrates for enzyme assays, coupled

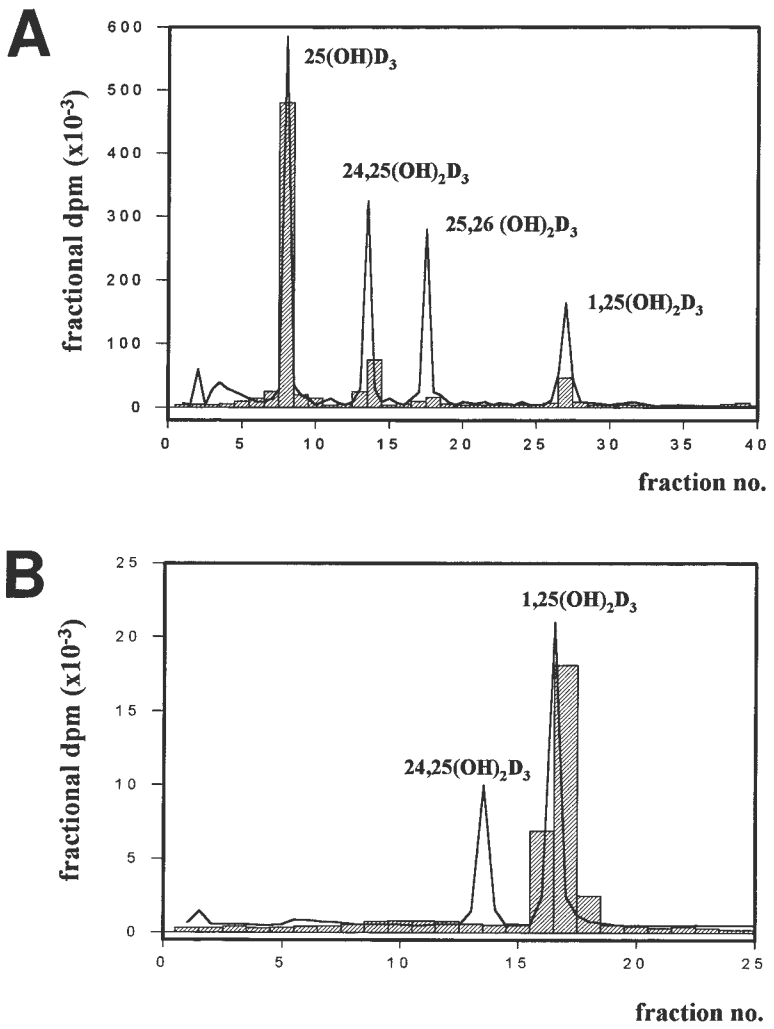

Fig. 2. HPLC analysis of ^{3}H-25(OH)D_3 metabolism in HKC-8 human kidney cells. **(A)** Straight-phase HPLC separation of radiolabeled vitamin D metabolites from cells incubated with 10 n*M* ^{3}H-25(OH)D_3 for 3 h at 37°C. **(B)** Reverse-phase HPLC separation of straight-phase HPLC fractions comigrating with authentic 1,25$(OH)_2D_3$ (*see* A). In both A and B, results are illustrated as fractional counts per minute (shaded boxes), plotted against an elution profile for standard vitamin D metabolites (line). Reproduced from Bland, R., Walker, E. A., Hughes, S. V., Stewart, P. M., and Hewison, M. (1999) Constitutive expression of 25-hydroxyvitamin D_3 1α-hydroxylase in a human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology* **140,** 2027–2034. © The Endocrine Society ***(11)***.

to either HPLC or TLC separation. In the case of the former, the HPLC column is usually connected to UV absorbance detector; TLC plates can be visualized with a simple UV light source. The latter, of course, relies on visual identification of bands of interest and manual measurement of migration distances, which requires relatively high concentrations of steroid hormone. Consequently, for the purposes of this chapter, protocols focus on the use of radiolabeled steroid substrates, which provide a more accurate and sensitive assessment of steroid metabolism (*see* **Note 1**).

3.1.2. Assay Design

3.1.2.1. Sample Choice and Preparation

Assays can be conveniently carried out using adherent cells grown in 24-well cell culture plates, as outlined in the two protocols described. With only slight changes, the technique can be adapted to cells grown in suspension. Although cells can be grown and treated in medium containing serum, analysis of steroid metabolism must be carried out under serum-free conditions to eliminate anomalies produced by binding proteins and endogenous steroids. Prior to the addition of labeled substrate, cells should be washed twice with serum free medium, then incubated with radiolabeled substrate for the required time.

The same techniques can be applied to tissue homogenates. However, when disrupted cells are used, it is important to mimic the microenvironment of the cell in terms of pH and temperature and to add any appropriate cofactors. The method of extraction also needs to be considered. For example, tissues can be disrupted by physical methods, such as sonication or homogenization. Chemical disruption by a lysis buffer can also be used, but the effect of the lysis buffer on enzyme activity must be considered. Depending on the cellular location of the enzyme of interest, it may be necessary to perform a subcellular fractionation, e.g., generate mitochondria or microsomes.

3.1.2.2. Enzyme Kinetics

Another vital component of enzyme analysis is an understanding of enzyme kinetics. A detailed examination of this subject is beyond the scope of this chapter, but is fully discussed in a number of books ***(9)***. Briefly, it is essential that reactions are linear regarding substrate concentration and time. Initial studies should use untreated cells for dose- and time-dependency analyses.

3.1.3. Analysis of Steroid Metabolites

Preparation of samples for TLC or HPLC is similar. Steroids and their metabolites are extracted from medium into an organic solvent, such as chloroform, dichloromethane, or ethyl-acetate (*see* **Note 2**). Following evaporation

of the organic phase, either by vacuum or by drying under air or nitrogen, samples are resuspended in solvent and applied to HPLC columns or TLC plates. HPLC requires a sample volume of 10–50 μL; TLC samples are normally applied in volumes of 50–100 μL.

3.1.3.1. Determination of Vitamin D Metabolites by HPLC

Caution should be taken when using any vitamin D metabolites, because these molecules are extremely labile. Stock solutions should be stored at –20°C, preferably under nitrogen and in the dark. Studies from the authors' group have highlighted the importance of extracting both the cells and growth medium present in the reaction mixture. Thus, it is essential to carry out a parallel set of incubations without the ^{3}H-25(OH)D$_3$, which can then be used to measure the concentration of proteins in cell monolayers. This will be used at a later stage to determine the level of vitamin D metabolism per mg of cellular protein. Remember that steroids stick to plastic, and therefore, where possible, glass containers should be used.

Although a protocol is not provided in this chapter, vitamin D metabolism can also be routinely determined by TLC ***(10,11)***.

3.1.3.1.1. Treatment of Cells

1. Grow cells to near confluence in a 24-well plate.
2. Change medium to serum free medium.
3. Add ^{3}H-25(OH)D$_3$. The authors routinely use a concentration of 3.75 n*M* (1.5 pmol ^{3}H-25(OH)D$_3$ in a volume of 400 μL serum-free medium per well), which is optimal for sensitive detection of 1α-hydroxylated metabolites in kidney cells.
4. Incubate cells with substrate for 4 h, or the required length of time.
5. Stop the reaction by freezing samples at –20°C. Because the authors are assaying the metabolites in both the medium and the cells, it's convenient to wrap the whole 24-well plate in aluminum foil and place directly into the freezer.
6. Parallel plates containing the samples for protein assay should also be frozen. The authors routinely perform the protein assay in the presence of our serum-free medium. However, if the serum-free medium used interferes with the protein assay, replace the medium with 1 mL of water before freezing the 24 well plates at –20°C.
7. Protein assay. Remove the plates from the freezer and allow to thaw. Remove cells from plate by scraping and release total cellular proteins by freeze-thawing. Assay proteins using any standard method.

3.1.3.1.2. Analysis of Tritiated Metabolites

To protect expensive silica or C-18 columns from being loaded with excess lipids, the authors recommend the inclusion of an initial clean up procedure as outlined in the following protocol for HPLC analyses. Alternatively, samples can be extracted and loaded directly onto the HPLC columns as described in **step 6**.

1. Remove cells from wells of culture plates by scraping and place medium and cells in glass tubes.
2. Add 2 mL of distilled water to the cell-medium mix. Freeze–thaw this mixture twice.
3. Prewash a C-18 Sep-Pak minicolumn with 5 mL HPLC-grade water, followed by 3 mL 70:30 (vol:vol) mixture of methanol:HPLC-grade water, then 5 mL acetonitrile.
4. Add the lysed cell–medium mixture to the prewashed Sep-Pak column. Elute vitamin D metabolites into a fresh glass tube with 3 mL acetonitrile:methanol (80:20, vol:vol).
5. Dry the eluent under nitrogen or vacuum.
6. Alternative extraction protocol:
   a. Remove cells from wells of culture plates by scraping and place medium and cells in glass tubes.
   b. Add 2 mL chloroform and 0.5 mL methanol to medium and cells and vortex.
   c. Centrifuge at 260*g* for 15 min.
   d. Remove upper, aqueous layer to waste.
   e. Dry the samples under nitrogen or vacuum.
7. Resuspend the eluted vitamin D metabolites in 50 µL running solvent (*see* **step 8**). Store at –20°C.
8. Vitamin D metabolites can be separated using a variety of silica HPLC columns. The authors routinely use Zorbax-sil (4.6 × 250 mm) columns (Anachem), which give high resolution with a fast elution time. Prior to the application of samples, equilibrate the HPLC column with 50 mL running solvent (hexane:methanol: isopropanol [92:4:4, vol:vol:vol]) at a rate of 2 mL/min. This should result in a constant and low pressure within the column.
9. Prior to sample analyses, standards should be analyzed to determine elution profiles. In the example shown in **Fig. 2A** the authors used a mixture containing 100 ng $25(OH)D_3$, and 50 ng each of $24,25(OH)_2D_3$, $25,26(OH)_2D_3$, and $1,25(OH)_2D_3$ in 50 µL running solvent, which was subsequently eluted for 20 min at 2 mL/min. The separation of these metabolites was determined by connection to a Waters Lambda Max 481 Liquid Chromatography Spectrophotometer set at a wavelength of 265 nm. The resulting peak profile was obtained using a standard chart recorder (**Fig. 2A**).
10. After optimizing standard separation (*see* **Note 3**), it is advisable to elute the column for a further 20 min to remove possible traces of metabolites.
11. Unknown samples can then be injected onto the column in 10–50 µL running solvent. The concentration of vitamin D metabolites in these preparations is much less than can be detected using spectrophotometry, but it is still useful to run the detector and chart recorder because this will alert the user to any problems with column contamination.
12. To detect the separation of radiolabeled vitamin D metabolites, it is necessary to collect fractions from the HPLC column every 30 s, which can be done directly

into scintillation vials if there is no further need for the sample. Otherwise, the eluents can be collected into glass tubes, then stoppered and stored at –20°C prior to further studies.

13. The radioactivity profile corresponding to the different metabolites of ^{3}H-25 (OH)D_3 can then be determined by adding 5 mL scintillant to each of the fraction tubes, followed by radioactive counting in a suitable scintillation counter.
14. A typical profile for distribution of radioactivity is shown superimposed on the standards in **Fig. 2A**.
15. Quantification of enzyme activity can be determined by converting the disintegrations per minute (DPM) of the tritiated metabolite corresponding to a particular standard peak to fmoles. This is dependent on the percentage conversion to a particular product. Thus, if the total number of DPM collected in a 20-min HPLC run is 100,000, and 5000 DPM coincided with the 1,25$(OH)_2D_3$ peak, the % conversion to 1,25 $(OH)_2D_3$ = 5% of the original amount of ^{3}H-25(OH)D_3. Taking into account the amount of cellular protein present, and the incubation period, it is possible to produce an activity value in pmol product/h/mg protein (as described in **Subheading 3.1.3.3.**).
16. The identity of the metabolites of interest can be confirmed using specific fractions collected from the Zorbax-sil column. These fractions are dried under nitrogen, and then reinjected on to a straight-phase HPLC column. Subsequent collection of new fractions should confirm that the radioactivity is still coincident with the standard. An example of this is shown in **Fig. 2B**, where an initial 1,25$(OH)_2D_3$ fraction from straight-phase HPLC has been reseparated on a Zorbax-ODS reverse-phase column and eluted with methanol:water (80:20, vol:vol) at 2 mL/min. As can be seen, the level of DPM recovered is relatively low, but radioactivity is still coincident with the 1,25$(OH)_2D_3$ standard.

3.1.3.2. Analysis of Androstenedione Metabolism in Intact, Adherent Cells by TLC

There are two main enzyme systems involved with the production of estrogen from androstenedione. Aromatase catalyzes the conversion of C-19 androgens to C-18 estrogens. Although it can convert testosterone to estradiol, the principle product of aromatization is estrogen. However, this can readily be converted to estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD). At least 11 isoforms of 17β-HSD have now been cloned ***(12)***, and these are able to interconvert estradiol and estrone, and androstenedione and testosterone. In vivo aromatase and the 17β-HSDs act in a coordinated fashion to regulate the interconversion of androgens and estrogens, as illustrated by **Fig. 3**. By incubating cells with tritiated androstenedione, one can follow the whole of this pathway. **Figure 4** is an example of a typical scan of a TLC plate. There are distinct peaks that represent testosterone (T), estradiol (E_2), androstenedione (A), and estrone (E_1) (*see* **Notes 4** and **5**).

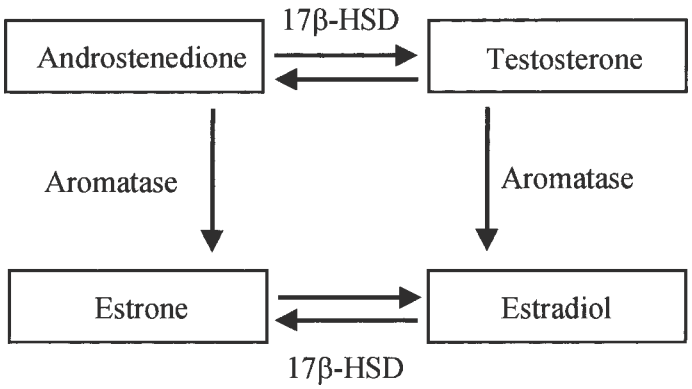

Fig. 3. Interconversion of androgens and estrogens by aromatase and 17β-HSD.

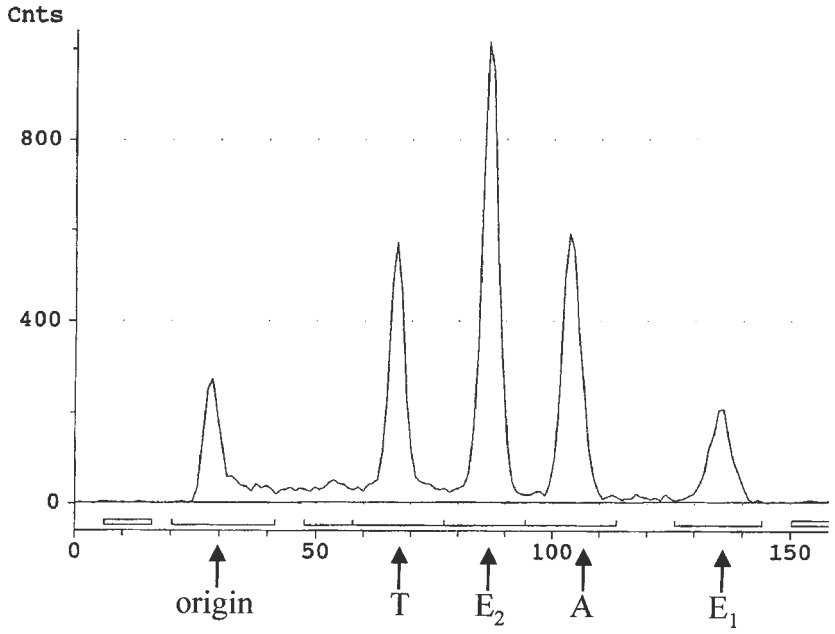

Fig. 4. Typical TLC trace of androstenedione metabolism showing peaks corresponding to testosterone (T), estradiol (E_2), androstenedione (A), and estrone (E_1).

3.1.3.2.1. Treatment of Cells

1. Grow cells to near confluence in a 24-well plate.
2. Change medium to serum free medium.

3. Add 40 n*M* androstenedione, one-third of which was labeled ^{3}H-androstenedione, to wells.
4. Incubate cells with substrate for 5 h or the required length of time.
5. Stop the reaction by removing the medium to glass tubes (approx 12 × 100 mm). Either assay medium immediately (approx 4 h), or store the samples at –20°C for analysis later.
6. Add 1 mL of water to the wells and store plates at –20°C for protein assay.
7. Protein assay. Remove cells from plate by scraping and release total cellular proteins by freeze–thawing. Assay proteins using any standard method.

3.1.3.2.2. Analysis of Tritiated Metabolites

1. Add 5 mL of chloroform to 1 mL medium and vortex.
2. Centrifuge at 260*g* for 15 min.
3. Remove upper, aqueous layer to waste (a vacuum pump with a glass Pasteur pipet attached is convenient).
4. Evaporate the chloroform. The authors routinely dry the samples under air at 56°C.
5. Prepare TLC plates by marking off positions of spots. These should be approx 2 cm from the lower edge of the plate and at 1.5-cm intervals across the plate. Twelve samples can be run on a standard TLC plate.
6. Once dried, resuspend the samples in 50 µL of chloroform, vortex (keep tubes stoppered to prevent evaporation), and spot onto the plate.
   Note: For correct identification of peaks produced, it is vital that radioactive standards of the expected products are run on each TLC plate.
7. Place in preequilibrated TLC tank containing chloroform:ethylacetate (4:1, vol:vol) as the solvent system.
8. Run plate until the solvent front is 1 cm below the top of the plate. Remove the TLC plate from the tank and allow to dry.
9. Analyze the conversion of tritiated androstenedione by using a TLC plate scanner, such as the Bioscan system 200 imaging TLC plate scanner (Bioscan, Edmonds, WA). If a TLC scanner is not available, samples can be analyzed by scintillation counting (*see* **Note 6**).

3.1.3.3. Analysis of Results

Levels of enzyme activity are normally expressed as amount of product produced/mg of protein present/h or min. The amount of protein/well can be calculated from the stored plates, and the % conversion of substrate to product is calculated by the plate scanner or determined from the scintillation counts (*see* **Note 6**). The activity of the enzyme can be calculated using the equation given below.

$$\frac{\text{pmol substrate per well} \times \%\ \text{conversion to product}}{\text{h of incubation} \times 100 \times \text{protein/well (mg)}} = \text{pmol/h/mg protein}$$

3.2. Steroid Hormone Receptor Binding Assays

3.2.1. Background

There are a number of techniques that can be used to examine the expression of steroid hormone receptors. Levels of mRNA and protein can be determined by Northern blot and Western Blot analysis, respectively, and both these techniques will show about the quantity of the receptor mRNA or protein, and will detect gross abnormalities in size. However, neither of these techniques tells anything about the function or the receptor, i.e., the ability of the receptor to bind ligand. Ligand-binding assays allow one to estimate the numbers of receptors present, but also allow one to determine the binding affinity of the receptor for the ligand.

The whole-cell binding assay outlined in **Subheading 3.2.2.** is based on the principal that steroids (in this case, the glucocorticoid, dexamethasone, and the mineralocorticoid, aldosterone), when added to cells, will enter the cells and bind to their cognate receptors. At a certain equilibrium time-point, all of the available receptors will be occupied by the steroid. The resulting ligand–receptor complexes will all be located in the nucleus. Thus, the assay is often called a "nuclear association" assay.

3.2.2. Analysis of Glucocorticoid Receptor and Mineralocorticoid Receptor Binding

Dexamethasone has a low affinity for the glucocorticoid receptor (GR) (dissociation constant, $K_d \cong 10$ nM) compared to the binding of aldosterone to the mineralocorticoid receptor (MR) ($K_d \cong 1$ nM). As such, the concentrations of labeled dexamethasone to be used will be between 1.56 and 50 nM, and for labeled aldosterone between 1.56 and 25 nM. Although steroid hormones bind with high affinity to their cognate intracellular receptors, they will also bind nonspecifically to other cellular proteins. To address this, it is always essential to include parallel binding assays, which include a large excess of unlabeled steroid. This will displace specifically bound radiolabeled steroid. Any remaining radioactivity is nonspecifically bound (i.e., background, *see* **Fig. 5A**).

Although dexamethasone is the GR ligand and aldosterone is the MR ligand, they are related corticosteroids, and both can bind to the GR and the MR. Therefore, it is prudent to include specific GR or MR antagonists, which will prevent nonspecific receptor binding. For example, inclusion of a 200-fold excess of RU752 in the GR binding assay will prevent dexamethasone binding to the MR, and likewise inclusion of a 200-fold excess of RU486 in the MR binding assay will prevent aldosterone binding to the GR.

3.2.2.1. Analysis of GR Binding

1. Prepare the following paired dilutions of labeled and unlabeled dexamethasone.

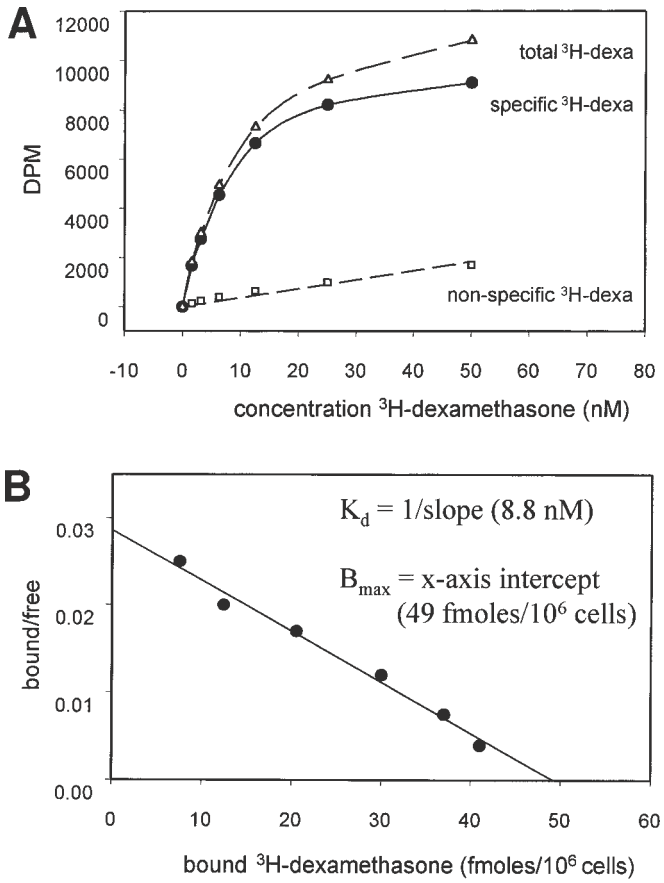

Fig. 5. ^{3}H-dexamethasone binding assay. **(A)** Saturation binding curve B. Scatchard plot **(B)**. Cells (10^6) were incubated with 1.56–50 n*M* ^{3}H-dexamethasone without or with a 200-fold excess of unlabeled dexamethasone.

Glass tubes	Unlabeled standards (μ*M*)	Labeled standards (n*M*)	Final conc. ^{3}H-dexamethasone (n*M*)
a.	200	1000	50
b.	100	500	25
c.	50	250	12.5
d.	25	125	6.25
e.	12.5	62.5	3.12
f.	6.25	31.25	1.56

2. Prepare two sets of glass tubes (a–f), one containing 10 μL labeled dexamethasone dilutions and 10 μL ethanol, and the other set containing 10 μL labeled and 10 μL unlabeled dexamethasone dilutions (200-fold excess).

3. Grow cells to 80% confluence in 75-cm^2 culture flasks.
4. Remove cells by trypsinization and wash twice in serum-free medium. Resuspend cells in serum-free medium to give 5×10^6 cells/mL.
5. Add 200 µL cells to each tube and incubate for 1 h at 37°C. Retain an aliquot of cells for protein determination. Binding can then be expressed per number cells or per mg cellular protein (*see* **Notes 7** and **8**).
6. Pellet cells and wash twice in ice cold PBS (700*g*, 4°C, 5 min).
7. Resuspend pellet in 500 µL lysis buffer, leave on ice for 10 min, and then pellet nuclei (700*g*, 4°C, 5 min). The resulting crude nuclear pellet should contain all of the liganded receptors.
8. Carefully aspirate supernatant from final cell pellet and resuspend in 200 µL cold PBS and 500 µL cold absolute ethanol.
9. Transfer to scintillation vials and count radioactivity.

3.2.2.2. Analysis of MR Binding

The above protocol describes the determination of GR binding assay. Mineralocorticoid binding can be determined in exactly the same way by substituting aldosterone ([1,2,6,7-^{3}H]aldosterone, 65 Ci/mmol, Amersham) for dexamethasone. Because the binding affinity of aldosterone for the MR is higher than that of the GR for dexamethasone (Kd of 1 n*M* vs 10 n*M*), the top concentration of aldosterone used can be reduced to 100 µ*M* unlabeled and 500 n*M* labeled.

3.2.2.3. Determination of Binding Affinity and Receptor Number

Scintillation counting of samples will give DPM for the two sets of samples; labeled dexamethasone only and labeled plus unlabeled dexamethasone. These data need to be transformed in order to determine the K_d and B_{max}. **Table 1** contains an example of the numbers obtained from a typical dexamethasone-binding assay. Columns A–D contain the raw data were used to calculate the numbers in columns E–H. These numbers can then be plotted as a binding curve (**Fig. 5A**) and in a linear form by Scatchard plot (**Fig. 5B**). From the Scatchard plot, it is possible to calculate the kinetics of dexamethasone binding. Binding affinity is represented by the dissociation constant (K_d), which is 1/slope of the line. Total binding capacity, or B_{max}, is the intercept with the *x*-axis (*see* **Note 9**).

To convert DPM to fmol, it is necessary to take account of the specific activity of the labeled steroid. As can be seen from **Table 1**, the figures in column F (fmoles) have been obtained by dividing the DPM by 222, a number that is dependent on the specific activity of the labeled steroid used and may vary with each batch of labeled steroid. For example the specific activity of the ^{3}H-dexamethasone used in this example was 100 Ci/mmol (100 pCi/fmol). 1 pCi of tritium is equivalent to 2.22 DPM; therefore, 100 pCi = 1 fmol = 222 DPM.

Table 1
Example of Data Obtained from a Typical Dexamethasone Binding Assay[a]

A fmol ^{3}H-DEX added per well	B ^{3}H-DEX (n*M*)	C Total DPM (labeled DEX only	D Labeled + unlabeled DEX (DPM)	E Specific DEX binding (DPM)	F Specific DEX bound (fmol/ 10^6 cells)	G Free	H Bound/ free
312	1.56	1818.0	153.0	1665.0	7.5	304.5	0.0250
625	3.12	2995.0	242.0	2753.0	12.4	612.6	0.0200
1250	6.25	4952.0	401.0	4551.0	20.5	1229.5	0.0170
2500	12.5	7313.0	653.0	6660.0	30.0	2470.0	0.0120
5000	25.0	9225.5	1011.0	8214.0	37.0	4963.0	0.0075
10000	50.0	10813.0	1711.0	9102.0	41.0	9959.0	0.0040

[a]The numbers in columns E–H were obtained using the following calculations: E = C–D; F = E/222; G = A–F; H = F/G.

4. Notes

1. If choosing a radiolabeled substrate, the positions at which the steroid is labeled are important. Ideally, the compound should be labeled at positions that are uninvolved in metabolism. If metabolism results in a loss of one or more radioactive labels, this decrease in radioactively must be compensated for in the calculations of product produced.
2. If setting up a new extraction procedure, one needs to ensure that both the substrate and metabolites are extracted with equal efficiency from the medium.
3. The HPLC separation profile shown in **Fig. 2A** is optimal, in that there is good separation of key metabolites while maintaining a tight peak profile for the key vitamin D metabolite, $1,25(OH)_2D_3$. This peak is smaller (but broader) than those obtained with $24,25(OH)_2D_3$ or $25,26(OH)_2D_3$, even though similar amounts of standard were applied. Standard profiles can be adjusted by either:
   a. Lowering/increasing the flow rate of the running solvent.
   b. Changing the solvent composition. For example, changing the solvent mix to hexane:methanol:isopropanol (94:3:3, vol:vol:vol) will increase the separation of the metabolites, but will also significantly increase the time needed to resolve $1,25(OH)_2D_3$ and the peak width of all the metabolites. In some cases, it is possible to elute the HPLC column with gradient solvent systems in which components, such as methanol and isopropanol, are gradually increased throughout a run. This improves the resolution of more polar species, such as $1,25(OH)_2D_3$, which are more readily retained on the HPLC column, because of their hydrophilic nature, and lack of affinity for organic solvents.
4. If samples do not run in a consistent manner, adding a spot of unlabeled steroid to each lane may be necessary to aid the movement of the sample up the plates.
5. If samples are difficult to separate by TLC, it may be necessary to use two different solvent systems. These can be used in two ways:
   a. Two-dimensional TLC. The first separation occurs as normal. The TLC plate is then rotated 90 degrees and placed in a different solvent system.
   b. Two-step TLC. This is the combination of running the TLC plate first in one solvent system, then placing the plate in a completely different system and allowing it to run a shorter distance up the plate. The authors have found this technique works well for the separation of estrone sulfate and estrone ***(13)***.
6. If a plate scanner is unavailable, the amount of conversion can be determined by scintillation counting. Identify the region of the plate that corresponds to the position of the compound of interest, which can be done by spotting concentrated samples of nonradioactive compounds on the TLC plate in each lane. The positions of the samples can then be visualized using UV light, and the region of the sample can be marked directly on the plate by pencil. Scrape the silica from the TLC plate into a glass tube containing 1 mL ethanol and store overnight at 4°C to allow steroids to elute. Centrifuge at 600*g* for 30 min and remove supernatant to a fresh tube. Add 0.5 mL ethanol to remaining silica, centrifuge as above, and pool the supernatants. Evaporate the ethanol and resuspend in 100 μL ethanol. Add to scintillant and count.

7. Depending on the level of expression of the receptor of interest in cells or tissue, one may need to alter the number of cells per assay tube (or amount of protein added, if tissue). For example, with low expression, one may need to increase cell numbers.
8. Although the authors have suggested incubation times and steroid concentrations, because each of these depends on a number a variables that are receptor type-, cell-, and tissue-specific, one may find that each of these parameters will need to be optimized for the cell system. This may be particularly important with respect to time required to reach binding equilibrium for a particular steroid hormone. For most steroids 1 h is sufficient, but it is important to test this prior to further work.
9. B_{max} can be converted from fmol/mg protein, or 10^6 cells, to actual receptors per cell by utilizing Avogadro's constant. In this case, a simple conversion factor would be to multiply by 602.2.

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12

Vitamin D_3 Analog Screening

Sami Väisänen, Sanna Ryhänen, and Pekka H. Mäenpää

1. Introduction

Human vitamin D receptor (hVDR) belongs to the superfamily of steroid receptors. The receptors are nuclear transcription factors that regulate gene expression in response to binding of their specific ligands. According to present knowledge, the molecular mechanism of vitamin D action involves ligand binding, which induces a conformational change into hVDR, which, in turn, enables transactivation. This can result in either activation or repression of gene transcription ***(1)***. In the search of potent vitamin D_3 analogs, it is reasonable to target the screening methods on the steps mentioned above.

The structure of 1α,25-dihydroxyvitamin D_3 differs from the other steroid hormones with respect to the opened B ring, resulting in a three-ring structure (**Fig. 1**). This structural feature along with the relatively long side chain, gives vitamin D higher flexibility compared with the other hormones in the superfamily ***(2)***. The synthetic vitamin D_3 analogs as well as the parent compound, 1α,25-dihydroxyvitamin D_3, have three structural features that determine their ligand-dependent transcriptional activity: the A ring, the side chain, and the D ring ***(2)***. The chemical and stereochemical modifications introduced into these parts of the structure may influence transcriptional properties of the ligand in several ways. Introduction of double bonds, triple bonds, or heteroatoms into the side chain or the D ring may either stabilize or destabilize the ligand increasing or decreasing its clearance rate. In addition, these modifications, as well as the side chain epimerization at C-20, may influence binding properties of the ligand to the receptor and ultimately change dimerization and DNA-binding properties of the receptor ***(3)***.

Methods that can be used to screen potent vitamin D_3 analogs can be categorized as in vivo and in vitro studies. Today, analogs are screened in vivo by

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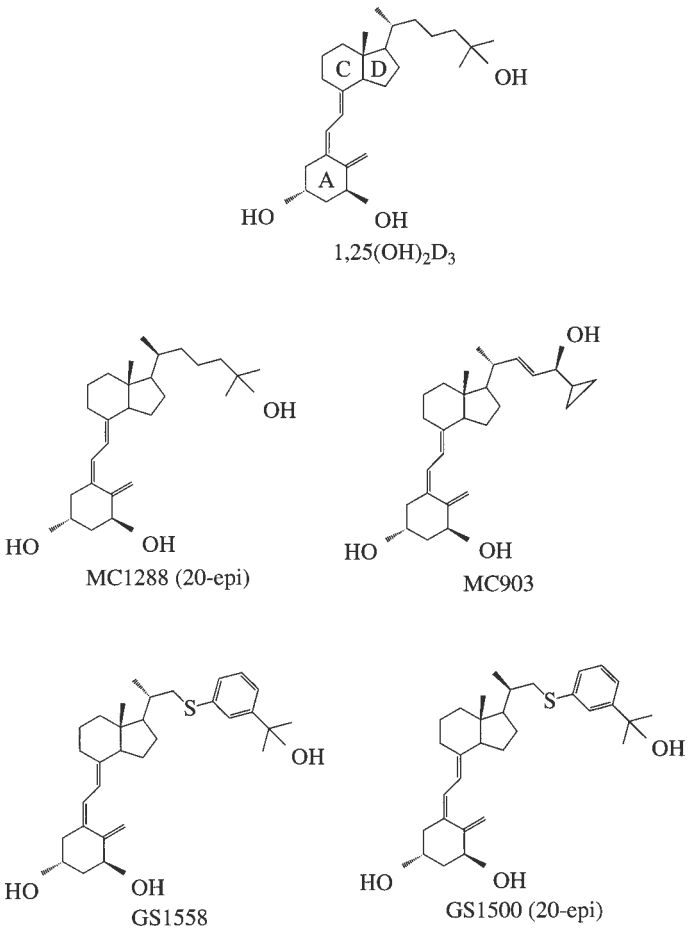


Fig. 1. Chemical structures of 1α,25-dihydroxyvitamin D_3 and its selected synthetic analogs. The A, C, and D rings are indicated.

examining their effects on plasma calcium levels in experimental animals, their growth inhibitory actions in cultured cells, and their anabolic effects on bone ***(3)***. This chapter describes in detail some of the most important in vitro screening methods targeted on ligand binding, VDR conformation, and biological activity. As model compounds, 1α,25dihydroxyvitamin D_3 and four of its analogs are used (kindly provided by Dr. Lise Binderup and Dr. Fredrik Björkling from Leo Pharmaceutical Products Ltd., Ballerup, Denmark), namely, MC1288, GS1558, GS1500, and MC903 (**Fig. 1**).

Although all methods described here are easy and fast to perform, these methods alone are not sufficient for complete analog screening, since in vivo

studies are also needed. Together, the in vitro and in vivo methods form a powerful tool for screening potent vitamin D_3 analogs. The methods described in this chapter, in principle, are also adaptable for analysis of other steroid receptors with their specific analog ligands.

2. Materials

2.1. Ligand-Binding Studies

1. 10 m*M* TENG buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylenediamine tetraacetic acid (EDTA), 0.02% NaN_3, 1 mg/mL gelatin. Slightly heat and mix with magnetic stirrer until gelatin is completely dissolved.
2. Dextran-coated charcoal (DCC)-buffer: 1% charcoal, 0.1% dextran T-70 in TENG buffer. Dissolve 50 mg dextran T-70 (Sigma, St. Louis, MO) into 40 mL TENG buffer. After the sugar has dissolved, add dH_2O to 50 mL. Add 500 mg charcoal and mix overnight with magnetic stirrer at 4°C. The mixture will stay in a refrigerator for at least 2 wk.
3. 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol, 179 Ci/mmol (Amersham, Buckinghamshire, England).
4. OptiPhase HiSafe 2 scintillation cocktail (Wallac, Turku, Finland).

2.2. Conformational Studies

1. hVDR cDNA inserted into plasmid pSP65 or other suitable vector with either SP6 promoter or T7 promoter.
2. TNT® Coupled Wheat Germ Extract System kit (Promega, Madison, WI).
3. L-[^{35}S]methionine, >1000 Ci/mmol (Amersham).
4. Trypsin (EC 3.4.21.4) (Sigma).

2.3. Biological Activity

1. Cell culture: Maintain human MG-63 osteoblastic osteosarcoma cells (American Type Culture Collection, Rockville, MD) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal calf serum (FCS), 2 m*M* L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin. Store the medium at 4°C. Heat-inactivate FCS (30 min, 56°C) and aliquot in 35–50 mL stocks. Prepare charcoal-treated FCS by gently mixing 1 part 10 g charcoal/1 g dextran in 100 mL DMEM with 5 parts of FCS, and heat-inactivate (45 min, 56°C) by stirring. Centrifuge at 15,500*g* for 10 min, filter sterilize the serum, and aliquot in 20-mL portions. L-glutamine is prepared in water, filter-sterilized, and aliquoted in stocks. Store all individual components at –20°C.
2. Guanidinium thiocyanate buffer: 4 *M* GuSCN, 25 m*M* Na-citrate, pH 7.0, 0.5% sarcosyl, 0.1 *M* β-mercaptoethanol. Dissolve 23.53 g GuSCN into 1.67 mL 0.75 *M* Na-citrate, pH 7.0, 2.5 mL 10% sarcosyl, and add distilled water to 50 mL. Slightly heat and mix with magnetic stirrer until dissolved. The buffer will stay at room temperature for up to 3 mo. Just before use, add β-mercaptoethanol to a final concentration of 0.1 *M*.

3. 10X 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer: 0.2 *M* MOPS, 50 m*M* NaAc, 10 m*M* EDTA, pH 7.0. Use autoclaved stock solutions, and mix 200 mL 1 *M* MOPS, pH 7.0, 16.7 mL 3 *M* Na-acetate, 20 mL 0.5 *M* EDTA, pH 8.0, and adjust the volume to 1000 mL with distilled water. 1 *M* MOPS, pH 7.0, stock solution: Dissolve 209.3 g MOPS in 800 mL distilled water using magnetic stirrer. Adjust the pH to 7.0 with 5 *M* NaOH and add distilled water to 1000 mL. Autoclave and store at room temperature, protected from light.
4. 20X standard sodium citrate (SSC) buffer: 3 *M* NaCl, 0.3 *M* Na citrate. Dissolve 175.3 g NaCl and 88.2 g Na citrate to about 800 mL with distilled water using magnetic stirrer. Adjust the pH to 7.0 and add distilled water to 1000 mL. Autoclave and dilute with distilled water to get 2X SSC (0.3 *M*/30 m*M*) and 10X SSC (1.5 *M*/0.15 *M*) solutions.
5. Hybridization solution: 50% formamide, 2X Denhardt's solution, 5X SSC, 1% sodium dodecyl sulfate (SDS), and 50 μg/mL denatured herring sperm DNA. Prepare immediately before the use.
6. Northern loading buffer: 50% glycerol, 10 m*M* Na-phosphate buffer, pH 7.0, 0.4% bromophenol blue. Store at –20°C.
7. Osteocalcin protein measurement: Human osteocalcin radioimmunoassay kit (CIS Bio International, Gif-Sur-Yvette, France).

3. Methods

3.1. Ligand-Binding Studies

The key step for the action of a vitamin D_3 analog is ligand binding. The ligand-binding affinity does not need to be high. In fact, many potent vitamin D_3 analogs have lower affinity to hVDR than 1α,25-dihydroxyvitamin D_3. Nevertheless, it is essential for the action of a vitamin D_3 analog that it binds to hVDR. To study whether the ligand of interest binds to the receptor, the easiest and fastest way to progress is to determine the maximal binding and compare it with that of a known compound, e.g., 1α,25-dihydroxyvitamin D_3. In this case, a radioactively labeled 1α,25-dihydroxyvitamin D_3 is compared with a large excess of the compound of interest and the reference compound and the results are compared (**Fig. 2A**). However, if more precise data are needed, the ligand-binding affinity can be studied either by classical Scatchard analysis (**Fig. 2B**) or by a competition assay with multiple concentrations of the ligand of interest (**Fig. 2C**). For the Scatchard analysis, the ligand of interest must be radioactively labeled. The labeling, however, is often time-consuming and expensive, and, therefore, it is often more feasible to use commercially available, radioactively labeled 1α,25-dihydroxyvitamin D_3 and to study the affinity by competition assay with nonradioactive analogs.

3.1.1. Scatchard Analysis

1. Prepare nonradioactive hVDR protein in vitro by the coupled wheat-germ-extract system, as described by the manufacturer (Promega).

2. Add 5 µL hVDR protein mixture together with 0.005–5 n*M* radioactive ligand diluted with ethanol in 500 µL Eppendorf tubes (*see* **Notes 1** and **2**). Add distilled water to a total volume of 20 µL and incubate for 30 min at 22°C.
3. Add 20-µL amounts of DCC-solution into 500-µL Eppendorf tubes, and centrifuge with Eppendorf microcentrifuge at full speed for 5 min. Discard the supernatant.
4. Pipet the mixture from **step 2** onto DCC pellet, and suspend well. Incubate for 10 min on ice. Centrifuge with Eppendorf microcentrifuge at full speed for 5 min. Carefully pipet the supernatant into scintillation counting tubes with 3 mL scintillation cocktail. Avoid pipeting the DCC, since it now contains free radioactivity. Contamination of the sample with DCC can be avoided by pipeting only 18–19 µL supernatant.
5. Count each sample for 300 s by scintillation counter.
6. Correct the results for background and plot as bound radioactivity against bound radioactivity vs free radioactivity (*see* **Note 3**). The plot should be a straight line with a slope of –1/Kd (**Fig. 2B**).

3.1.2. Competition Analysis

1. Prepare nonradioactive hVDR protein in vitro by the coupled wheat-germ-extract system as described by the manufacturer (Promega).
2. Add 5 µL hVDR protein mixture, together with 0.5 n*M* 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol, 179 Ci/mmol (Amersham), diluted with ethanol into 500 µL Eppendorf tubes (*see* **Notes 1** and **2**). Add increasing concentrations (e.g., 0.001–10 n*M*) of the competing ligand and distilled water to a total volume of 20 µL and incubate 30 min at 22°C.
3. Add 20-µL amounts of DCC-solution into 500-µL Eppendorf tubes and centrifuge with Eppendorf microcentrifuge at full speed for 5 min. Discard the supernatant.
4. Pipet the mixture from **step 2** onto DCC pellet and suspend well. Incubate for 10 min on ice. Centrifuge with Eppendorf microcentrifuge at full speed for 5 min. Carefully pipet the supernatant into scintillation counting tubes, with 3 mL of scintillation cocktail. Avoid pipeting the DCC, since it now contains the entire free radioactivity. Contamination of the sample with DCC can be avoided by pipeting only 18–19 µL supernatant.
5. Count each sample for 300 s by scintillation counter.
6. Correct the results for background and plot as bound radioactivity (e.g., cpm%) against log [competitor] (*see* **Note 3**). The plot should be a sigmoidal line (**Fig. 2C**). From the plot, the EC_{50} and IC_{50} values can be determined. In many cases, these values give sufficient data when they are compared with the reference compound 1α,25-dihydroxyvitamin D_3. However, if the dissociation constants are needed, they can be calculated from EC_{50} values. The results of competition assays should not be analyzed by linear methods, such as the Scatchard analysis, but rather by nonlinear regression. However, Scatchard plots can be used to display the results, since straight lines are more easily comprehensible than rectangular hyperbolas.

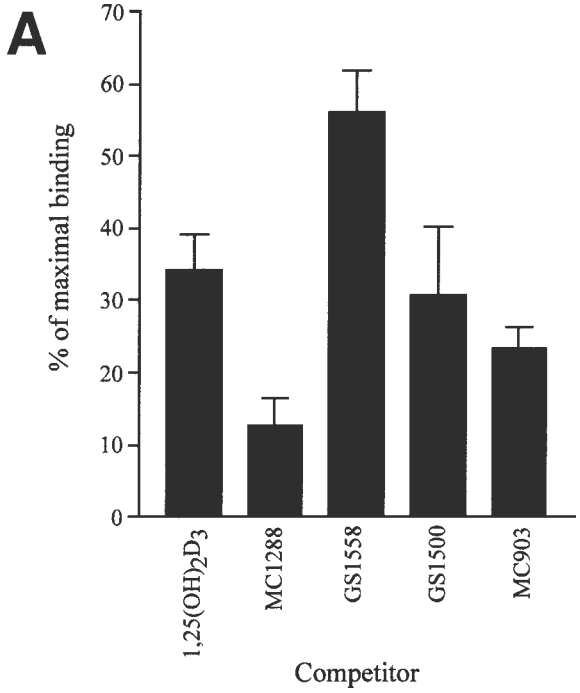

A
70
60
50
40
30
20
10
0
% of maximal binding
1,25(OH)2D3
MC1288
GS1558
GS1500
MC903
Competitor

Fig. 2A.

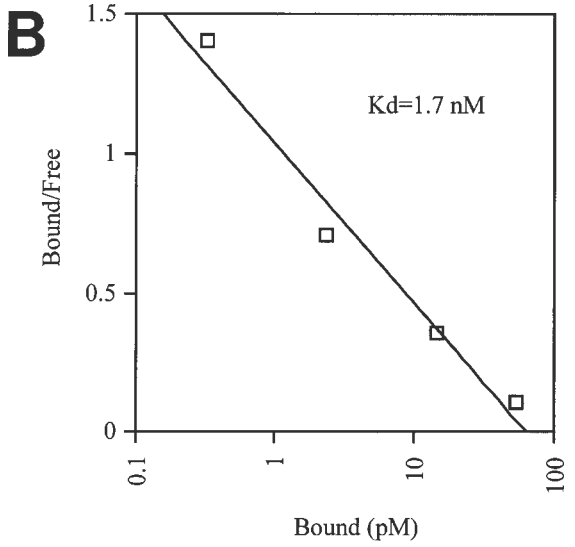

B
1.5
1
0.5
0
Bound/Free
Kd=1.7 nM
0.1
1
10
100
Bound (pM)

Fig. 2B.

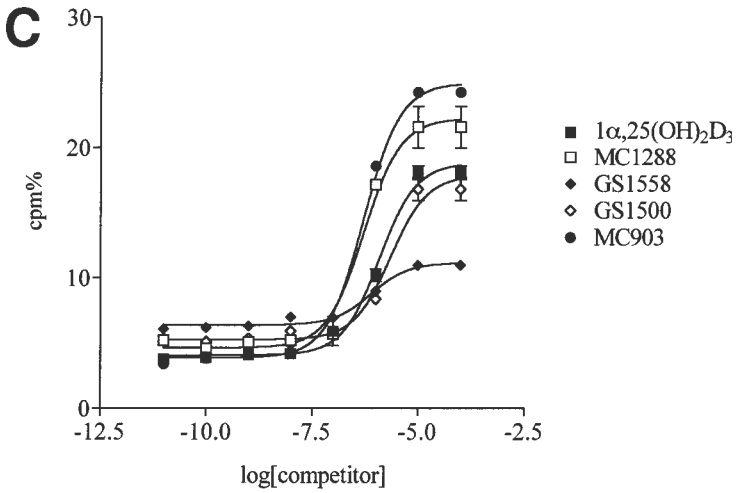


Fig. 2. Ligand binding studies. **(A)** Maximal binding by competition assay. 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol (0.5 n*M*) was compared with a 1000-fold excess of nonlabeled 1α,25$(OH)_2D_3$ and its synthetic analogs. **(B)** Scatchard analysis of 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol. **(C)** Competition analysis of 1α,25$(OH)_2D_3$ and its synthetic analogs. 1α,25-dihydroxy[26,27-methyl-^{3}H]-cholecalciferol (0.5 n*M*) was compared with nonlabeled 1α,25$(OH)_2D_3$ and its synthetic analogs (10^{-5}–10^{-12} *M*).

3.2. Conformational Studies

Today, conformational studies of hVDR are seldom used to screen potent vitamin D_3 analogs. However, the ligand-binding-induced conformation of the receptor has an important role in the biological actions of the analogs. In fact, the final conformation of the hVDR–ligand complex directly influences heterodimerization with retinoid X receptor and the binding of transactivators, and, finally, the biologic response of the analogs. Most of the potent vitamin D_3 analogs that are presently in preclinical or clinical development (MC1288, EB1089, GS1500) ***(3–6)*** are able to stabilize the ligand binding domain of in vitro translated hVDR against limited proteolytic digestion by trypsin ***(7)*** (**Fig. 3**).

The easiest way to begin the conformational studies of the hVDR protein (or any other nuclear receptor protein) is in vitro translation of the receptor. An obvious advantage of this method is that, when the cDNA of protein of interest is available, the protein itself will also be available. Further, no special knowledge is needed concerning methods of cell culture, protein expression, or protein purification. The disadvantage of this method is that post-translational modifications of the protein, e.g., phosphorylation, probably do not occur.

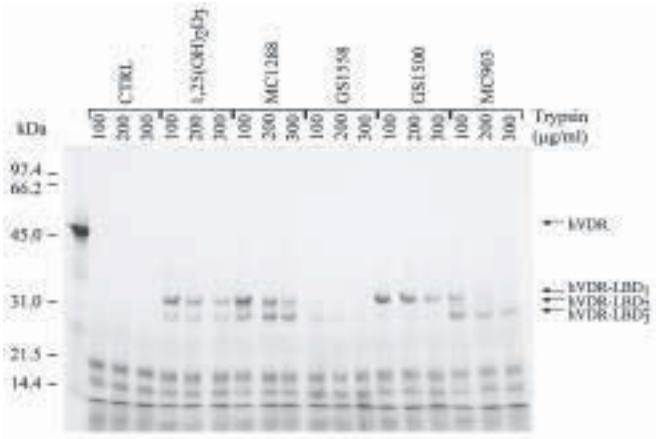


Fig. 3. Conformational analysis of hVDR. [^{35}S]methionine-labeled, in vitro-translated hVDR was treated for 30 min at 22°C with 1α,25(HO)$_2$ D$_3$ and its synthetic analogs before being exposed to increasing amounts of trypsin (100–300 μg/mL). Limited proteolytic digestion produces three main fragments (hVDR-LBD$_1$, hVDR-LBD$_2$, and hVDR-LBD$_3$). After limited proteolytic digestion, the receptor occurs predominantly in the conformation hVDR-LBD$_1$, which is suggested to be agonistic conformation.

Thus, one must keep in mind that the in vitro-translated protein may differ from its native counterpart in its conformation and action.

3.2.1. Limited Proteolytic Digestion

1. Prepare L-[^{35}S]methionine >1000 Ci/mmol (Amersham)-labeled hVDR protein in vitro by the coupled wheat-germ-extract system. as described by the manufacturer (Promega).
2. Add 5 μL hVDR protein mixture with 1 μ*M* of nonradioactive ligand, diluted with ethanol into 500-μL Eppendorf tubes, and incubate for 30 min at 22°C (*see* **Notes 1** and **2**).
3. Add 0–300 μg/mL trypsin and incubate for 10 min at 22°C.
4. Stop the digestion by adding fivefold SDS loading buffer and boiling for 5 min.
5. Separate the digestion products by 15% SDS-polyacrylamide gel electrophoresis.
6. Dry the gel and autoradiograph overnight at –80°C (*see* **Notes 4** and **5**). Change the exposure time if needed.

3.3. Biological Activity

Human MG-63 cells are osteoblast-like osteosarcoma cells. These cells are capable of expressing genes of the most differentiated osteoblast phenotype, including that of osteocalcin. Osteocalcin is a bone specific protein, which is synthesized by osteoblasts, and is regulated by 1α,25(OH)$_2$D$_3$. In human

MG-63 cells, *osteocalcin* gene activity can be used as an indicator of both transcriptional (**Fig. 4A,C**) and translational effects (**Fig. 4D**) of vitamin D_3 and the analogs. The effects of vitamin D_3 compounds on MG-63 bone cells can be studied as a function of time, and/or by using different concentrations of the ligand. To study the duration of the effect after withdrawal of the ligand, the cells are treated with the selected vitamin D_3 compound for a short period of time (6 h). The medium is then changed into a new medium and the cells are cultured without the ligand for the next 120 h.

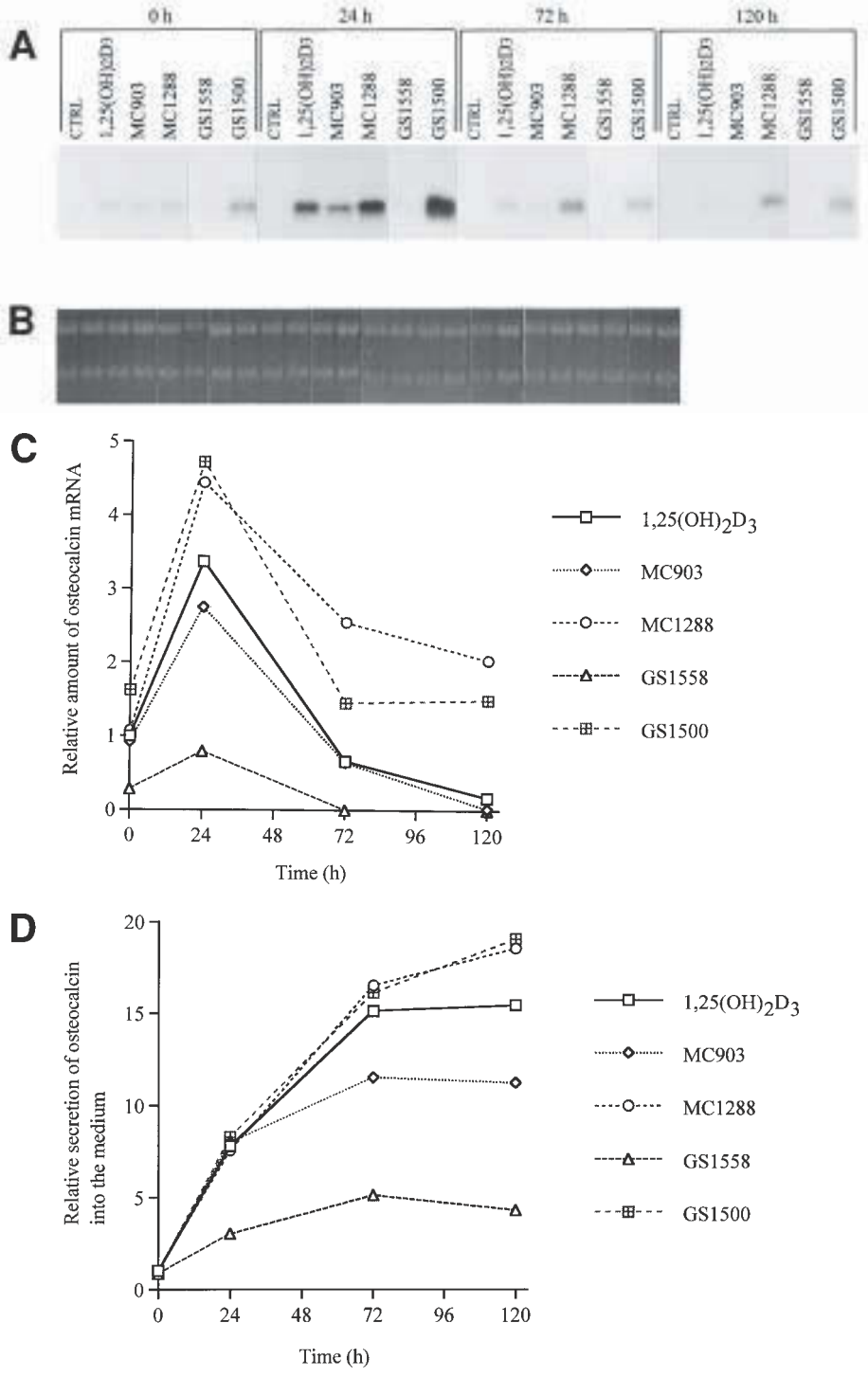
3.3.1. Duration Studies for Vitamin D_3 Compounds

Culture MG-63 cells at 37°C under 5% CO_2 in the respective culture media described above. Dilute 1α,25$(OH)_2D_3$ or the analogs in ethanol before adding to the cell cultures and treat the control cultures with 0.1% ethanol.

1. Seed MG-63 cells at 3–4 × 10^5 cells/60-mm plate. Use at least triplicate plates in each treatment.
2. After 24 h, replace the medium by fresh medium containing 2% charcoal-treated FCS (*see* **Note 6**).
3. Allow the cells to grow for 24 h, then treat the cells with either 10 n*M* calcitriol or the analogs for 6 h.
4. After the 6-h pretreatment period, collect the cells and the media for the first time-point (0 h). For the later time-points, replace the medium by fresh medium containing 2% charcoal-treated FCS and culture the cells without the vitamin D_3 compounds for up to 120 h.
5. After different time-points (e.g., 24 h, 72 h, and 120 h), collect the media for radioimmunoassay (RIA) analysis and the cells for Northern blot analysis by rubber policeman.

3.3.2. Northern Analysis for Detection of Osteocalcin mRNA Levels

1. Isolate total cellular RNA from cultured cells using the guanidinium thiocyanate method according to Chomczynski and Sacchi ***(8)*** or by using commercial kits (e.g., Promega, Qiagen, or Sigma) or other methods.
2. Quantify RNA by diluting 2.5 µL in 0.5 mL sterile H_2O and reading the A_{260} and A_{280}. Use a quartz microcuvet. (Store RNA at –70°C.)
3. Adjust the volume of each RNA sample (5–20 µg) to 5 µL with sterile water, then add 15.5 µL of a mixture that contains 2 µL 10X MOPS buffer, 3.5 µL 12.3 *M* formaldehyde, and 10 µL deionized formamide. Mix and incubate for 30 min at 50°C.
4. Prepare 1% agarose/formaldehyde gel: Dissolve 1.0 g agarose in 72 mL water and cool to <60°C, then add 10 mL of 10X MOPS running buffer and 18 mL 12.3 *M* formaldehyde. Pour the gel and allow it to settle. Remove the comb, place the gel in the gel tank, and add sufficient 1X MOPS running buffer to cover to a depth of ~1 mm.


A
0 h
24 h
72 h
120 h
CTRL
1,25(OH)2D3
MC903
MC1288
GS1558
GS1500
B
C
Relative amount of osteocalcin mRNA
Time (h)
1,25(OH)2D3
MC903
MC1288
GS1558
GS1500
D
Relative secretion of osteocalcin into the medium
Time (h)
1,25(OH)2D3
MC903
MC1288
GS1558
GS1500

5. Add 2 µL Northern loading buffer to the samples, centrifuge briefly, and load the samples onto the gel. Electrophorese the RNA samples under denaturing conditions in a 1% formaldehyde/agarose gel.
6. At the end of the run, visualize the RNA with a UV transluminator after staining with ethidium bromide. The 28S and 18S ribosomal RNAs should appear as discrete bands at approx 5.3 and 2.0 kb, respectively. Photograph the gel (**Fig. 4B**).
7. Rinse the gel with several changes of sufficient deionized water to cover the gel. Transfer the fractionated samples from the gel to a nitrocellulose membrane by upward capillary transfer. After transfer, UV-bake the filter for 5 min.
8. Prepare a DNA or RNA probe labeled to a specific activity of $>10^8$ cpm/µg, with unincorporated nucleotides removed. In osteocalcin hybridizations, use a 5'-end-labeled 40-residue oligonucleotide, 5'-CCAACTCGTC ACAGTCCGGA TTGAGCTCAC ACACCTCCCT-3', complementary to human mRNA sequence coding for amino acids 20–32 of the mature osteocalcin ***(9)*** (*see* **Note 7**).
9. Wet the membrane in 2X SSC. Prehybridize the filter in hybridization tube or bag, and add ~1 mL formamide prehybridization/hybridization solution/10 cm^2 of membrane. Incubate for 2–12 h at 42°C. Denature the probe by heating in a water bath, or block for 10 min at 100°C and transfer to ice. Pipet the probe into tube or bag, and continue the incubation for 24 h.
10. Wash the membrane: Pour off the hybridization solution and add 5X SSC. Incubate with rotation for 15 min at 42°C, change wash solution, and repeat the incubation. Replace wash solution to 1X SSC 0.1% SDS and incubate for 30 min at room temperature. Remove the final wash solution and seal the membrane in the plastic bag. Perform autoradiography overnight at –80°C.
11. Scan the resultant autoradiogram with a densitometer (**Fig. 4A**) and correct these scanning values relative to scanning values of RNA loading controls (**Fig. 4B**) (*see* **Note 5**). Compare the results of analog treatments with $1\alpha,25(OH)_2D_3$ treatments (**Fig. 4C**).

3.3.3. Radioimmunoassay for Detection of Osteocalcin Protein Levels

Osteocalcin is secreted into the culture medium. After each treatment and time-point, the secreted osteocalcin is measured by radioimmunoassay (**Fig. 4D**).

1. Centrifuge the collected media briefly (20,000*g* for 10 s).
2. Measure osteocalcin protein concentrations from the media according to the manufacturer's instructions (CIS Bio International).

Fig. 4. *(opposite page)* Maintenance of *osteocalcin* gene expression in MG-63 cells, after treatment with $1\alpha,25(OH)_2D_3$ or its analogs. Medium was changed after the 6-h pretreatment (0 h), and the incubation was continued for 120 h without further hormone additions. **(A)** Osteocalcin Northern blots after the different treatments. **(B)** Gel stained with ethidium bromide before transfer, showing the 28 S and 18 S rRNAs. **(C)** Osteocalcin mRNA levels quantified by densitometric scanning of the autoradiograms. **(D)** Relative osteocalcin secretion into the medium. The symbols in C and D represent osteocalcin mRNA and protein levels, relative to the 6-h pretreatment with $1\alpha,25(OH)_2D_3$.

3. Using the standards, draw a standard curve and read the sample values directly from the curve.
4. Compare the results of analog treatments with $1\alpha,25(OH)_2D_3$ treatments (**Fig. 4D**).

4. Notes

1. Buffering of the VDR in ligand binding and conformational studies: The basic protocols do not necessarily demand any specific buffering. However, if other factors, e.g., DNA or transcriptional cofactors, are added to the system, it is recommended that a binding buffer is used. For this purpose, the binding buffer for the electrophoretic mobility shift assay is suitable.
2. Dilution of analogs: Usually, the analogs are stored in 2-propanol and diluted with ethanol for ligand binding and conformational studies. However, ethanol has a vitamin D_3 antagonistic nature and competes with the analogs. Therefore, it is sometimes useful to dilute the analogs with dimethyl sulfoxide ***(10)***.
3. Ligand-binding assays: To have reliable results, the concentration of the radioactive ligand used in the analyses must be high enough. The amount of bound radioactivity must not exceed 70% of the total radioactivity added to the reaction. The incubation time of the ligand treatment should not be shorter than 30 min. Otherwise, it is possible that the equilibrium will not be reached.
4. Conformational studies: Sometimes, especially with fresh radioactive label, there are 2–3 nonspecific zones visible in the autoradiographed SDS-polyacrylamide gel that can disturb the results. These fragments probably result from free label or breakdown of the in vitro-translated protein, and can be avoided by treating the samples with DCC solution between **steps 2** and **3** in **Subheading 3.2.1.**
5. If Phosphoimager is available, it is recommended to use it in determining the band intensities.
6. Perform all cell culture experiments in a medium containing 2% charcoal-treated FCS to eliminate effects of endogenous steroid hormones.
7. In hybridizations, it is possible to use DNA, RNA, and oligonucleotide probes that are either radioactively (e.g., ^{32}P) or nonradioactively (e.g., digoxigenin) labeled. The different probes may require different hybridization conditions (buffer, temperature, washing, detection).

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IV

Protein Interaction Assays

13

Application of Green Fluorescent Protein to the Study of Dynamic Protein–Protein Interactions and Subcellular Trafficking of Steroid Receptors

Steven K. Nordeen, Paul R. Housley, Yihong Wan, and Richard N. Day

1. Introduction

The green fluorescent protein (GFP) from the jellyfish, *Aequoria victoria*, converts blue light to green fluorescence when expressed in intact cells and transgenic animals, and has proven to be a powerful tool for biological and medical research. This chapter describes the application of spectrally distinguishable variants of GFP to the investigation of steroid hormone receptor action. Topics that are covered include the design of GFP–receptor chimeras, the expression of GFP-fusion proteins in cells in culture, the detection of the GFP-tagged receptors in living and fixed cells, and the use of GFP-variants to study the colocalization and interaction of steroid receptors and other proteins. Specifically, the authors describe the application of GFP-tagged steroid receptors to assess issues in receptor trafficking and receptor interaction with coactivator proteins. The latter approach employs fluorescence resonance energy transfer (FRET), a technique that effectively permits a 100-fold enhancement beyond the inherent resolving power of the light microscope.

1.1. Properties of GFP

Aequoria victoria GFP is a 27-kDa protein possessing a tripeptide chromophore buried within a β-barrel. The chromophore is formed by a post-translation oxidation and cyclization reaction involving the tripeptide serine^{-65}, tyrosine^{-66}, and glycine^{-67} (*see* **Note 1**). Illumination of GFP with near UV-wavelength light results in green light emission ***(1)***. GFP retains its fluorescent properties when expressed as a fusion to other proteins, allowing it to be used

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as a label for localization of proteins in intact cells. GFP fluorescence is more resistant to photobleaching than fluorescein, but can be photobleached, given a sufficiently intense excitation source; a property that has been exploited in studies of protein trafficking in the Golgi, for example ***(2)***. Mutagenesis of *Aequoria* GFP has generated color variants ranging from blue to yellowish green, and some of these variants can be readily distinguished by fluorescence microscopy. The combination of two different color variants allows the behavior of independent fusion proteins to be monitored in the same intact cell. The major limitation to the detection of the GFP-fusion proteins is autofluorescence from the living cells in culture and from the tissues of transgenic organisms ***(3)***. This background signal can be substantial when the excitation source required is in the near-UV wavelengths. Because of this, the mutant GFPs that have red-shifted excitation spectra and increased brightness compared to wild type GFP represent a substantial improvement for the detection of fusion proteins in living cells.

1.2. Variant GFPs

Mutation of residues in and around the chromophore and elsewhere in the protein has resulted in GFP variants with improved brightness and spectral characteristics, while maintaining the advantageous properties of GFP, such as its stability. Mutation of the chromophore serine residue to threonine resulted in GFP^{S65T}, which has both excitation and emission spectra shifted to longer (red-shifted) wavelengths, allowing fluorescence to be detected with the same light source and filters as used for fluorescein. Moreover, this mutant chromophore formed more efficiently and exhibited a 4–6-fold improvement in brightness compared to wild type ***(4–6)***. Further improvements in protein expression were achieved by optimizing codon usage for mammalian cell expression.

Changing the chromophore tyrosine residue to histidine resulted in a blue shift in the emission wavelength ***(7,8)***. Even with other optimizing mutations, however, the blue fluorescent protein (BFP) yields a substantially lower signal intensity than GFP^{S65T}, and it is sensitive to photobleaching. However, because of the substantial separation of the emission maximum of BFP and GFP^{S65T} (445 and 511 nm, respectively), BFP has utility for use in conjunction with GFP for co-localization and in FRET experiments. Many of the limitations inherent in the use of BFP were overcome by the development of a cyan (blue-green) color variant (mutant W7) ***(8)***. This variant resulted from mutation of the chromophore tyrosine to tryptophan, combined with mutation of several other residues within the surrounding β-barrel structure. When illuminated at 433 nm, the cyan fluorescent protein (CFP) fluoresces with a peak emission at

475 nm. This mutant is brighter and more resistant to photobleaching than BFP. Further, this mutant can be combined with another color variant, the yellowish fluorescent protein (YFP, mutant 10C) ***(8)*** in two-color imaging studies. The YFP variant is the most red-shifted of the *Aequoria* GFP proteins currently available with a peak emission at 527 nm. It is also the brightest protein, but it is more susceptible to photobleaching than its green counterpart ***(9)***. The combined use of CFP and YFP avoids some of the shortcomings inherent in studies with BFP, and this pair can be used as partners in FRET studies ***(10)***. The spectral overlap of these proteins, however, does constrain their use in this application.

1.3. GFP-Steroid Receptor Chimeras

The use of GFP as a molecular tag requires that both GFP and its fusion partner remain functional. The folding properties and stability of GFP make it particularly robust as a fusion partner in chimeric proteins. GFP has retained its fluorescent properties when fused to any number of other proteins and has also done so in a wide variety of different organisms and subcellular environments. Although neither N- or C-terminal truncations can be tolerated (with the exception of a few C-terminal amino acids [AAs]) ***(11)***, fusions can be done successfully at either the C- or N-terminus of GFP. The authors have made GFP–steroid receptor chimera proteins, in which GFP has been fused to the N-terminus of the steroid receptor (glucocorticoid and progesterone receptors) or the C-terminus (estrogen receptor). In all cases, the receptors were tested for their ability to enhance expression from reporter genes whose promoters possess the appropriate response elements (discussed further in **Subheading 3.1.**). Some investigators have used linker sequences to separate GFP and the receptor ***(12)***, although the authors have not found it necessary to interpose sequences in addition to those arising from the restriction sites or linker sequences in the vectors. GFP has been used to tag each of the members of the steroid receptor subfamily: the glucocorticoid ***(13–19)***, progesterone ***(12)***, estrogen ***(20–22)***, androgen receptor ***(23,24)***, and mineralocorticoid receptors ***(25)***. In addition, GFP chimeras of several members of the larger nuclear receptor family have been expressed, including the thyroid hormone ***(26)*** and vitamin D ***(27)*** receptors, and representatives of the so-called orphan receptors, as well ***(28)***. The authors have also used GFP tagging to study the localization of receptors that are themselves chimeras between related members of the nuclear receptor family (glucocorticoid and progesterone receptors). This has permitted the localization of the receptor domains that determine the differential steady-state distribution of the glucocorticoid receptor (cytoplasmic) and the progesterone receptor (nuclear) in the absence of ligand ***(29)***.

The domain structures of both steroid receptors and GFP have heretofore permitted retention of both GFP and overall receptor function, but more subtle influences of the GFP tag on receptor function have not been tested. The behavior of the chimeric protein must be evaluated in each case with respect to the parameters under study to certify that the chimeric protein is recapitulating behavior of the wild type protein.

1.4. Applications of GFP to Mechanisms of Steroid Receptor Action

1.4.1. Receptor Trafficking

Many of the investigations that have employed GFP-tagged steroid receptors have been directed to the study of steroid receptor localization and trafficking. Steroid receptors conduct intricate interactions with complexes of molecular chaperones in the absence of ligand and with various coactivators or corepressors, upon binding agonist or antagonist ligands, respectively. Moreover, receptors are continuously shuttling between cellular compartments in both the presence and absence of ligand ***(30–32)***. GFP-tagging has already made contributions to the study of receptor localization and trafficking, and other studies promise to address understudied areas, such as the kinetics of DNA binding and occupancy by receptors ***(33)***. The ability to follow GFP fluorescence in the unfixed cell also opens avenues to follow time-dependent changes in a single cell.

GFP itself is distributed throughout the nucleus and cytoplasm of the cell. Its small size and compact folding mean it is well below the exclusion limit of nuclear pores, and therefore enters the nucleus readily in the absence of nuclear-targeting signals. GFP does not appear to possess signals that target it to any specific cellular compartment, although it is excluded from certain compartments, such as the nucleoli. The neutral properties of GFP with respect to localization, make it an ideal partner for the study of trafficking properties of steroid receptors and the mechanisms involved.

1.4.2. Colocalization

Availability of spectrally distinct variants of GFP permits the assessment of two (or potentially more) GFP-tagged proteins simultaneously in the same cell. Increasing evidence indicates that transcription factors are localized to discrete domains with the nucleus. The authors have expressed BFP-tagged estrogen receptors along with putative estrogen receptor interacting proteins, the homeodomain transcription factor, Pit-1, or the coactivator, GRIP-1 (TIF-2). Merging of the individual BFP and GFP images indicate that GFP-Pit-1 and hER-BFP have a distinct, but overlapping pattern of distribution within the

nucleus ***(20)***, coexpression of hER-BFP and GFP–GRIP-1 results in a complete overlap of expression. The distribution of GFP–GRIP-1 is different without coexpression of estrogen receptor ***(21)***. These results suggest a direct interaction of estrogen receptor and GRIP-1, but optical resolution is insufficient to make a more direct inference. Fortunately, there is a means to circumvent these physical limitations.

1.4.3. Fluorescence Resonance Energy Transfer

Although colocalization experiments can yield data suggestive of direct interaction between proteins in vivo, preferable would be a direct demonstration of physical interaction between proteins. The resolution of the optical microscope is physically limited by the wavelength of visible light and can indicate proximity on the scale of about 250 nm. The diameter of an average-size globular protein and hence the approximate resolution required to demonstrate interaction, is on the order of one-fiftieth of that (~5 nm). Spatial resolution on this order can be achieved by conventional light microscopy using the technique of FRET. Energy transfer occurs when a donor fluorophore transfers excitation energy directly to an appropriately positioned acceptor fluorophore. The subsequent sensitized emission of the acceptor is detected in the light microscope. The efficiency of energy transfer varies inversely with the sixth power of the distance separating the donor and acceptor fluorophore, effectively limiting FRET to a range of 2–10 nm ***(8,10)***.

FRET requires a substantial overlap in the emission spectrum of the donor with the absorption spectrum of the acceptor, and that the fluorophores be appropriately positioned relative to one another. Regarding the variant forms of GFP, BFP can serve as a donor for the enhanced GFP ***(8,20–21,34–38)***; likewise, the cyan variant can serve as a donor for the yellow variant ***(10)***. Thus, if two proteins tagged with the appropriate donor and acceptor GFP pairs are expressed in the same living cell, and acceptor emission is detected following excitation at the donor excitation wavelength, then this is indicative of close apposition, implying a physical interaction between the protein pair. Detection of FRET and, by inference, interaction of the pair of labeled proteins, can be done in real time in the context of the living cell. The authors have detected FRET between BFP-tagged human estrogen receptor and the putative coactivator, GRIP-1, tagged with GFP.

These preliminary results with fluorescent protein-tagged steroid receptors are extremely exciting. However, there are a number of limitations and drawbacks to the system. One ramification of this is that the absence of a signal is not informative, i.e., does not necessarily imply the lack of interaction. These technical limitations are discussed in **Subheading 3.6.** Nonetheless, the poten-

tial for investigating details and dynamics of receptor–protein interactions in vivo in real time makes the application of GFP-tagged proteins, in conjunction with FRET, a powerful and attractive approach.

2. Materials

2.1. GFP-Receptor Chimeras and Other GFP-Tagged Fusion Proteins

1. phER-GFP, encoding a fusion of GFP^{S65T} with the C-terminus of the human estrogen receptor α.
2. phER-BFP, encoding a fusion of BFP with the C-terminus of the human estrogen receptor (*see* **Note 2**).
3. pGFP-PR, encoding a fusion of Enhanced GFP (*see* **Note 3**) with the N-terminus of the B isoform of the human progesterone receptor.
4. pGFP–GR, encoding a fusion of Enhanced GFP (*see* **Note 3**) at amino acid 9 of the human glucocorticoid receptor.
5. GFP-mGR, encoding a fusion of enhanced GFP (*see* **Note 3**) with the N-terminus of the mouse glucocorticoid receptor.
6. pGFP–GRIP-1, encoding a fusion of enhanced GFP (*see* **Note 3**) with the N-terminus of the steroid receptor coactivator, GRIP-1.
7. pGFP-Pit-1, encoding a fusion of a GFP^{S65T} at the N-terminus of the transcription factor, PIT-1.
8. pGFP-9AA-BFP, encoding a fusion of GFP^{S65T} and BFP, coupled by a 9 amino acid linker (*see* **Note 4**).

2.2. Expression of GFP-Tagged Steroid Receptors by Transfection

2.2.1. General Materials

1. Tissue culture (TC) hood and CO_2 incubator.
2. Culture medium and serum.
3. Sterile tissue culture dishes and pipets.
4. Hemacytometer.
5. Glass cover slips. Sterilize by autoclaving in glass Petri dish or foil.
6. Glass microscope slides.

2.2.2. Diethylaminoethyl–Dextran Transfection

1. 20 mg/mL diethylaminoethyl (DEAE)–dextran solution: Dissolve DEAE–Dextran (Pharmacia) in culture medium at 37°C overnight. Filter-sterilize, aliquot, and store at 4°C.
2. 100 m*M* chloroquine solution: Dissolve chloroquine in H_2O. Filter-sterilize, aliquot, and store at –20°C.
3. Master mix: 1/1000 vol of 100 m*M* chloroquine plus 1/100 vol of 20 mg/mL DEAE–dextran in complete culture medium. Final concentration of chloroquine and DEAE–dextran, 100 μ*M* and 200 μg/mL, respectively.

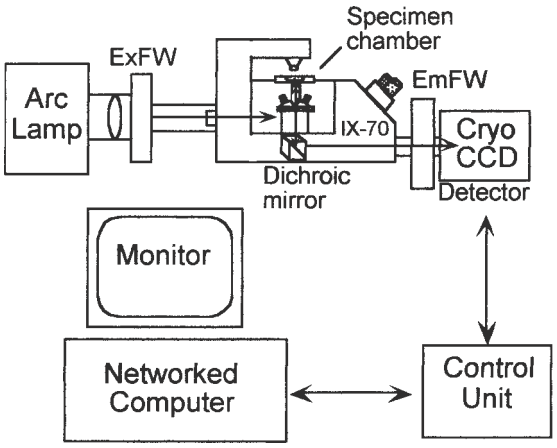

Fig. 1. Schematic diagram of an imaging system.

4. Transfection mix: Add 1 μg of each expression plasmid per mL master mix. Mix thoroughly.
5. 10X shock buffer: 1.37 *M* NaCl, 50 m*M* KCl, 60 m*M* glucose, 7 m*M* Na_2HPO_4, 210 m*M* HEPES. Adjust pH to 7.10, filter-sterilize, and store at 4°C.
6. Dimethylsulfoxide (DMSO).
7. DMSO shock solution: one-tenth vol 10X shock buffer plus 15/100 vol DMSO in sterile H_2O (*see* **Note 5**).

2.2.3. For Electroporation

1. Electroporation cuvets: Either 0.2 or 0.4 cm gap.
2. Dulbecco's calcium–magnesium free phosphate-buffered saline (PBS).
3. Electroporation unit (e.g., BTX electrocell manipulator 600, San Diego, CA).

2.3. Receptor Localization

1. Aside from standard laboratory materials, the only specialty equipment required for detecting the subcellular localization of the GFP–receptor fusion proteins expressed in single living cells is a quality epifluorescence microscope. The use of a high-quality imaging system, which has matched apochromatic optics, high numerical aperture, water immersion objectives, and provides uniform illumination of the specimen will improve the sensitivity and resolution of images obtained from living cell preparations. A schematic diagram of an imaging system used for some of these studies is shown in **Fig. 1**.

   The system consists of an inverted microscope equipped with epifluorescence and transmitted illumination (IX-70, Olympus America, Melville, NY). A 1.2 numerical aperture ×60 aqueous-immersion objective lens is used to acquire the image of the living cells in the specimen chamber on the microscope stage (*see* **Note 6**). The epifluorescent light source is a 100 W mercury-xenon arc lamp

(Hamamatsu, Middlesex, NY). Excitation filter wheel (ExFW), emission filter wheel (EmFW), and neutral density (ND) filter wheels (Ludl Electronic Products, Hawthorne, NY) are interfaced with a Silicon Graphics, (SGI) networked computer system. Dichroic mirrors are installed in filter cubes (Omega Optical, Brattleboro, VT). The detector is a liquid nitrogen-cooled charge coupled device (Cryo CCD camera, CH260, Photometrics, Tucson, AZ). The SGI based Isee software (Inovision, RTP, Raleigh, NC) is used both to integrate the operation of the camera and filter wheels, and to control image acquisition and image processing.

2. In some cases, better resolution may be obtained using a laser-scanning confocal microscope (*see* **Subheading 3.3.**). For some studies on receptor localization and trafficking, the authors have used a Bio-Rad MRC 1024 instrument.
3. Useful small equipment includes hanging drop slides (Fisher no. 12-560 or equivalent) and fine-tip tweezers for handling etched grid cover slips (Bellco no. 1916-92525).

3. Methods

3.1. Design of GFP–Receptor Chimeras

There are several important considerations when constructing vectors to express GFP–steroid receptor chimeras. Because proteins may be fused to either the N- or C-terminal end of GFP, an educated guess about which fusion will work best may be made, based on the known domain structure of the protein of interest. In practice, however, it may be useful to prepare both the N- and C-terminal fusions, and there are commercially available expression vectors with multicloning sequences to facilitate this. Moreover, having both fusions can be particularly useful for FRET studies since energy transfer is a function of both orientation and separation distance of the fluorophore pair. The authors have used chimeras in which GFP has been fused at or near the N-terminus of the progesterone receptor or the glucocorticoid receptor. These fusions are as effective as the WT receptor, as assessed by their ability to induce transcription of hormone-dependent reporter genes ***(29)***. When GFP was fused with the N-terminus of the estrogen receptor the resulting fusion protein was unable to induce transcription. Indeed, the GFP–ER protein acted as a dominant negative estrogen receptor. The authors' studies with estrogen receptor therefore have employed estrogen receptor α with fluorescent proteins fused at the C-terminus of the receptor ***(20,21)***. Curiously, an N-terminal estrogen receptor fusion has been used by others ***(22)***, although this protein appeared to be constitutively active.

Another important consideration is whether a linker sequence is to be used to separate the protein of interest from GFP. For the steroid receptor-GFP chimeras employed, the authors have not found this to be necessary. However,

in some cases, this may help by allowing both the tagged protein and the fluorophore to fold properly. The specific design and properties of the fluorescent protein-tagged receptors are more critical when the fusion proteins will be employed in FRET studies. A 15-amino acid long α helix is approx 2.2 nm in length, and FRET is sensitive to the separation (and orientation) of the donor and acceptor fluorophore pair with a useful range of 2–10 nm. Concerning this, the insertion of proline or glycine residues into a linker sequence to disrupt secondary structure or promote flexibility may be beneficial. Alternatively, it may be possible to dispense with some of the sequence of one GFP fusion partner, thereby repositioning the fluorescent protein more favorably with respect to the fluorophore tag on the interacting protein.

3.2. Expression of GFP-Tagged Steroid Receptors by Transfection

It is critical to test the function of fusion proteins in as many ways as possible. Fluorescence microscopy of cells expressing the chimeric proteins will show that the expressed protein fluoresces, demonstrating that GFP is intact and folds properly. This will also reveal the subcellular localization of the fusion protein, and comparison with cells expressing the GFP variant alone may be useful as a control for signal intensity and localization. In addition, comparison with cells in the same microscope field, which do not express the fusion protein, will provide an internal control for the autofluorescence background.

Western blotting of expressed proteins extracted from the transfected cells, using antibodies against either GFP or the protein of interest (or, for a more rigorous test, both), will demonstrate that the expressed protein is full-length. This is critical for proteins in which the GFP is at the N-terminus, in which case, truncation of the tagged protein would not be detected by fluorescence microscopy. Antibodies directed against GFP are commercially available.

In addition, it is critical to identify a method that directly demonstrates that the GFP-fusion protein retains the functions of its endogenous counterpart. For GFP fusions to the steroid receptors, the ligand-dependent regulation of an appropriate reporter gene construct in transient transfection assays is an important demonstration of function. Dose response studies can assess whether hormonal agonists are activating receptor-dependent transcription at the expected concentrations. Additional functional assays that can be performed include a direct assessment of binding of radiolabeled ligand and electrophoretic mobility shift assays using nuclear extracts prepared from transfected cells to demonstrate that the chimeric receptors bind to DNA with appropriate specificity.

A variety of methods are available to introduce purified fusion proteins or expression vectors into cells. The method of microinjection provides a means to introduce purified proteins, in vitro-transcribed mRNA, or DNA into cells in culture. This method can result in high expression levels and may be the method of choice for primary cell cultures. However, the approach is technically challenging, and the number of cells that can be injected for each experiment is limited. Transient transfection techniques, on the other hand, can yield high numbers of cells expressing the proteins of interest. A high efficiency of transfection is important for assessing fusion protein function by the methods described above, but it is less critical for actual imaging studies. Individual cells expressing GFP, representing less than 1% of the total cell population, can be easily detected by fluorescence microscopy. The method of transfection is dictated by the cell type used, and the conditions need to be optimized. In some cases, maximum efficiency may be sacrificed for superior preservation of morphology. Two methods of transfecting GFP–steroid receptor expression vectors into target cells are outlined.

3.2.1. Electroporation

1. Aliquot expression vector DNAs to sterile electroporation cuvets, either 0.2 or 0.4 cm gap may be used, but conditions for electroporation vary with the type of cuvet. Use empty vector as filler DNA, to keep total DNA constant. Typical amounts of DNA used in electroporation are 10–30 μg/cuvet.
2. Wash the cell monolayer with PBS.
3. Briefly treat the cells with trypsin (0.05%) in balanced salts with 0.53 m*M* ethylenediamine tetraacetic acid.
4. When cells begin to release from the surface of the flask, resuspend in culture medium containing serum.
5. Wash the cells twice by centrifugation in Dulbecco's Ca–Mg-free PBS.
6. Resuspend the cells thoroughly at a concentration of 1×10^7 cells/mL, and aliquot to the electroporation cuvets. The volume is critical (and depends on the type of cuvet used), and the cell suspension aliquot should be as uniform as possible.
7. Mix gently and place the cuvet in the electroporation unit. Pulse the cells at the desired voltage and capacitance.
8. Immediately remove the cuvet and dilute the cells in phenol red-free tissue culture medium containing serum (*see* **Note 7**).
9. Inoculate the cells onto sterile cover glass in 35-mm culture dishes by adding, dropwise, to the center of the cover glass. Let the cell suspension sit undisturbed for approx 20 min so the cells can begin to attach, then gently flood the culture dish with medium and place the cultures in the incubator.
10. Electroporation conditions for some cell lines used are provided in **Table 1** as reference for starting points to empirically determine the conditions that are suit-

Table 1
Electroporation Conditions

Cell type	Voltage	Capacitance
293 Human embryonic kidney	200	1200
Chinese Hamster Ovary K1	250	1400
COS 1 monkey kidney	250	1200
GH3 rat pituitary	180	1000
GHFT1-5 mouse pituitary	220	1200
HeLa human cervical carcinoma	250	1200
Rat 1 rat fibroblast	250	1200

able for a particular cell line and culture conditions. The following conditions were determined using the BTX electroporator at a maximum voltage setting of 500 V, resistance setting R3 (48 ohms), using 0.2-cm gap cuvets with 400 μL cell suspension in Ca–Mg-free Dulbecco's PBS. The typical pulse durations obtained under these conditions were 9–10 ms.

3.2.2. DEAE–Dextran Transfection

1. Day 1: Using sterile forceps, place 2–3 sterile glass cover slips on each 60-mm tissue culture dish.
2. Rinse Ltk^- or glucocorticoid receptor negative E82.A3 fibroblasts with serum-free medium or other balanced salt solution.
3. Add trypsin; incubate at room temperature until cells are released from the plate. Add cell culture medium with serum to inhibit trypsin.
4. Count cells in hemacytometer.
5. Plate 1.4×10^6 cells on each 60-mm tissue culture dish, adding enough complete medium to bring to 3–4 mL.
6. Use sterile pipet tips to push down the any glass cover slips that are floating. Shake or tap dish to ensure even plating of the cells. Do not swirl.
7. Incubate cells 16–24 h in 5% CO_2 at 37°C prior to DEAE–dextran treatment.
8. Day 2: Prepare master mix. 1 mL/60-mm dish will be required.
9. Prepare transfection mix.
10. Remove medium from dishes to be transfected.
11. Add 1 mL transfection mix to each dish.
12. Incubate at 37°C, 5% CO_2 for 2 h.
13. Aspirate the transfection mix.
14. Add 1 mL DMSO shock solution to each 60-mm dish.
15. Incubate at room temperature for precisely 6 min.
16. Aspirate the shock solution.
17. Immediately add 3 mL complete culture medium to each 60-mm dish.
18. Incubate at 37°C, 5% CO_2 for 44–48 h (*see* **Note 8**).

3.3 Receptor Localization and Trafficking

1. Transfected cells attached to the nonetched opposite surface of a gridded cover slip are imaged as live cells in culture medium, using confocal microscopy (*see* **Note 9**). Cover slips are gently grasped at the edge with fine-tip tweezers, inverted onto a hanging drop slide with culture medium filling the concavity, and examined under mercury lamp illumination. The use of the ×10 objective allows a large field containing several cells to be inspected. The desired cells are identified by their position in the grid under visible light, then scanned using the laser (*see* **Note 10**). Scan living cells at low laser power (<10%). This avoids the potential for photobleaching GFP and minimizes heating and denaturing the cellular contents.
2. Several serial Z scans on different cells should be done to unambiguously determine the cellular compartments containing the GFP–receptor fusion protein (*see* **Note 11**). This is especially important if the distribution pattern is not exclusively cytoplasmic or nuclear. After scanning is completed, the image is saved as a digital file, and the cover slip is carefully removed from the slide, inverted, and returned to culture medium and the incubator.
3. For experiments examining the effects of ligands on receptor localization, it is important to culture transfected cells in steroid-free medium, supplemented if necessary with charcoal-stripped serum. The ligand is added on cover slips to the plates containing the cells, incubated for the desired period, and the same cells previously scanned are located again using the grid and scanned.
4. One interesting use of this method is to examine the intracellular trafficking kinetics of steroid receptors, particularly glucocorticoid receptor. In hormone-free cells, the native glucocorticoid receptor and the GFP–GR are exclusively cytoplasmic. Upon exposure to a glucocorticoid agonist at 37°C, the GFP–GR translocates rapidly to the nucleus with a $t_{1/2}$ of ~5 min. If the cells are washed free of ligand, the GFP–GR exports slowly from the nucleus. Complete cytoplasmic redistribution may take 8–18 h, depending on the cell type. This property allows sequential temporal scans to be taken on the same individual cell, and the kinetics of nuclear export can be determined after quantifying total nuclear signal intensity (*see* **Note 12**).

3.4. Colocalization of Receptors and Other Proteins

The coexpression of proteins tagged with either the GFP and BFP or CFP and YFP spectral variants allows two independent fusion proteins to be monitored in the same living cell by fluorescence microscopy. Appropriate filters are required to discriminate between the emission of BFP or CFP from that of the coexpressed GFP^{S65T} or YFP. Often, narrow bandpass emission filters will improve the discrimination of fluorescence signals above the wide spectrum cellular autofluorescence signal. Moreover, because of the broad excitation and emission spectra of these fluorophores, it is important to select an excitation

Table 2
Suggested Filter Combinations

GFP variant	Absorbance maximum (nm)	Emission maximum (nm)	Filter component		
			Exciter	Dichroic	Emitter
GFP^{S65T}	489	511	470/40	495	515/30
BFP	381	445	365/25[a]	400	460/50
CFP	433	475	436/20	455	480/40
YFP	513	527	500/20	515	530LP

[a]To minimize excitation of GFP^{S65T} in dual color imaging.

filter that has a minimal coincidental excitation of the coexpressed partner. Filter sets that are designed specifically for the detection of the different color variants of GFP are now commercially available. **Table 2** shows suggested filter combinations.

Because of the bleaching characteristics of both BFP and YFP, it is important to scan and acquire images of cells expressing the GFP or CFP partners first before looking at the more sensitive fluorophores. Using transient cotransfection of expression vectors encoding proteins tagged with either GFP^{S65T} or BFP, the authors find good agreement in the expression levels for both fusion proteins. Therefore, scanning the field for cells expressing a certain level of green fluorescence is often a good predictor of the BFP expression level in that cell, and avoids photobleaching of the BFP. Achieving protein expression levels that will allow the use of some neutral density filtration will reduce the spectral scattering from the excitation light source, lower the autofluorescence background, which is substantial at the wavelengths used to excite BFP, and will also help to control the photobleaching of BFP.

1. Insert the cover glass with transfected cells attached into an appropriate chamber that fits the stage of the microscope being used. There are a number of different types of chambers available to fit microscope stages, and temperature-controlled chambers are commercially available.
2. If the culture medium is exposed to room air, use a culture medium that is buffered to maintain pH in room air.
3. Using the GFP filter set, scan the field to find healthy cells that are expressing reasonable levels of the GFP-fusion protein. Acquiring a bright field image of the selected cells is useful for determination of subcellular localization of the expressed protein.
4. Acquire the fluorescence image of GFP. Note that there be no saturated pixels in the acquired fluorescence image, since signal level cannot be determined in this

situation. Adjust camera integration time, neutral density filtration, and focus to optimize the GFP image, then acquire the final image.
5. Save the GFP image for further processing using the appropriate computer software.
6. With the excitation shutter closed, switch to the BFP filter set and appropriate dichroic mirror.
7. Using the same focal plane, acquire the BFP image. The authors find that increasing camera integration time to approximately twofold of that used for the GFP image is often sufficient to acquire the dim BFP signal.
8. Save the BFP image for further processing.
9. To process the images, computer software, which allows for background subtraction and creation of red, green, blue images from the acquired gray-scale images, is useful. The authors use the Inovision ISEE software for this purpose. In addition, different dichroic mirrors often produce images that are slightly out of register with one another. Software that allows registration correction will help, if the images are to be merged to demonstrate colocalization of the tagged proteins.

3.5. Detecting Interaction of Steroid Receptors and Coactivators by FRET

FRET microscopy detects the sensitized emission from the acceptor fluorophore, which is the result of energy transferred from an appropriately positioned donor. The demonstration of sensitized acceptor fluorescence at the excitation wavelength for the donor provides evidence that the distance separating the protein partners is on the order of 2–10 nm, and implies physical interaction. BFP can donate excitation energy to GFP^{S65T}, and the CFP variant can serve as a donor for YFP. As with colocalization studies described above, appropriate filters are required to discriminate between donor, acceptor, and sensitized acceptor (FRET) emission. Filter sets that are designed specifically for the detection of FRET using the BFP–GFP^{S65T} or the CFP–YFP pairs are commercially available. A number of different controls are critical for FRET studies.

3.5.1. Controls

1. In each experiment, some cells must express donor alone, and some express acceptor alone in order to determine the relative contribution of spectral crosstalk in the acquired images. Collect images of cells expressing a range of either donor or acceptor using each of the three filter combinations (donor, acceptor, FRET).
2. It is also important to have cells expressing a pair of proteins in the same subcellular compartment that should not physically associate. For this purpose, the authors have used a GFP protein with a nuclear localization signal and BFP-tagged nuclear proteins. This allows the acquisition of images using the FRET filter set, which show a range of background levels.

3. Critical for FRET is a positive control. For this purpose, the authors developed a fusion protein that contains GFP^{S65T}, coupled directly to BFP through a 9 amino acid linker. Expression of this fusion protein in cells results in sensitized acceptor emission that is approx twofold greater than donor emission ***(21)***.

The methods for FRET imaging are similar to those described above for colocalization studies. Again, the photobleaching characteristics of both BFP and YFP should be considered in acquiring the cells to be studied.

3.5.2. FRET Methods

1. Scanning the field for cells expressing a certain level of either GFP or CFP should predict the level of the BFP or YFP partners for a selected cell without bleaching the fluorophores. Acquire and save the fluorescence image of GFP (CFP). This provides a reference image for acceptor expression.
2. With the excitation shutter closed, switch to the BFP filter set and appropriate dichroic mirror, and acquire and save the donor image at the same focal plane. Immediately close the excitation shutter if this is not automatic.
3. Switch to the acceptor filter set that excites the donor and collects acceptor emission, and acquire and save the acceptor image at the same focal plane using the identical conditions and integration time used for the donor image.
4. To process the images, background subtract the donor and acceptor images. The simplest way to determine the relative levels of donor and acceptor emission is to ratio the two images and display the ratio of the image intensities using a look-up table. Alternatively, the background-subtracted donor and acceptor images can be combined into a single mosaic image, and look-up table is applied to indicate the pixel-by-pixel Fl signal intensity in the side-by-side images. For direct comparison, profiles of the signal levels can be plotted by both donor and acceptor fluorescence. Comparison of many similar cells expressing the fusion-protein partners will allow the average sensitized acceptor signal to be determined.

Comparison of these results with those from the control experiments described above will allow demonstration that sensitized emission has occurred, providing evidence for protein–protein interactions. These controls and the experimental images must be acquired under the same conditions. The levels of control-protein expression should be as similar as possible to those acquired under the experimental conditions.

3.6. Limitations of FRET Microscopy

FRET microscopy has the potential to become a routine analytical tool for detecting protein–protein interactions in living cells. There are, however, several important limitations to the application of this technology.

1. Spectral crosstalk. A major limitation of FRET microscopy performed with two independent proteins expressed in living cells, is accounting for spectral crosstalk

for both donor and acceptor fluorophores. This occurs because of the broad excitation and emission spectra of these fluorophores, resulting in donor-emission overlapping into the acceptor filter and excitation of the acceptor at the donor-excitation wavelengths. Computer software that can be calibrated to account for spectral crosstalk between donor and acceptor pairs was recently developed, and a quantitative method for determining FRET efficiency was introduced ***(38)***.

2. Geometry. The failure to detect FRET from a pair of labeled proteins does not imply that the protein partners are not physically associated. There are many potential reasons why interacting protein partners may fail to produce FRET signals. Because energy transfer is critically dependent on both the distance separating the fluorophores and their relative orientation, the conformations that are adopted by the interacting proteins may prevent the fluorophores from aligning properly.
3. Relative concentration of the fluorophores. The detection of FRET between independently expressed proteins is also limited by uncertainty of the relative concentrations of the expressed donor and acceptor fusion proteins.
4. Endogenous homologs. The endogenous counterparts of the labeled proteins also will interact with the expressed GFP chimeras, competing for potential productive interactions. This can be minimized by expressing the labeled proteins in heterologous cell types that lack the endogenous proteins, or by expressing the labeled protein partners in excess of the endogenous proteins. However, excessively high level of expression of proteins that are localized similarly within the cell, but not directly interacting, could potentially allow FRET to occur by diffusion. As with any approach involving the expression of proteins in living cells, artifacts that arise from overexpression of the fusion proteins are a concern. Control experiments with labeled proteins that colocalize, but that should not physically interact, can be used to assess the contribution of diffusion to measured FRET signals.
5. Photobleaching. Photobleaching of fluorophores during measurement. As indicated earlier, both BFP and YFP are sensitive to photobleaching (*see* **Subheading 1.2.**)
6. Sensitivity of detection equipment. Fluorescence imaging of living cells, especially when using BFP, requires the detection of very low levels of light, typically on the order of 10^{-4}–10^{-8} foot-candles. The video imaging detector needs to be sufficiently sensitive to detect these low-level signals. Further, since the detector is where the majority of signal amplification occurs, the dark current noise of the detector should be minimized. For FRET microscopy it is especially important to select a detector with high quantum efficiency (less noise), high sensitivity, and a fast readout rate. These are characteristics that can be found in both charge-coupled device cameras and photomultiplier tubes. The slow-scan, cooled, charge-coupled device cameras have reduced readout noise, and are capable of prolonged exposures, to detect low photon fluxes. Alternatively, photomultiplier tubes have high sensitivity, stability, low noise, rapid response (on the order of subnanoseconds) and very large dynamic range (>1 million-fold). The camera that the authors have used for some of the FRET imaging described

here was a slow-scan, liquid-nitrogen-cooled charge-coupled device camera with a back-thinned, back-illuminated imaging chip (CH260, Photometrics, Tucson, AZ).

4. Notes

1. The post-translational oxidation and cyclization steps are autocatalytic, but demand an aerobic environment and time. Cyclization is a slow event, requiring several hours to occur, which must be considered when attempting to image newly synthesized GFP or GFP fusion proteins.
2. The authors have found superior detection of the BFP derived from the vector phBFP (in which the BFP is derived from the mutant P4-3 ***(8)*** with humanization of the first 58 codons), compared to that commercially available from Clontech.
3. Clontech markets enhanced GFP expression vectors for making either N-terminal or C-terminal fusions. Vectors with polylinker cloning sites are available for each of the three reading frames.
4. Before initiating energy transfer experiments using proteins tagged with BFP^{Y66H}, BFP^{Y154F}, and GFP^{S65T}, both positive and negative controls must be designed to calibrate the microscope system. The expression of a tethered GFP-BFP fusion protein under the precise experimental conditions provides a way to optimize the optics, filters, and detectors for the discrimination of fluorescence signals and energy transfer. Of equal importance is a negative control to verify that these signals result from energy transfer and are not the result of channel overlap ***(39)***. The authors have used the expression of noninteracting GFP- and BFP-fusion proteins, both of which are targeted to the cell nucleus as a negative control for FRET imaging.
5. The dissolution of DMSO into an aqueous buffer is exothermic. Prepare the DMSO shock solution at least 30 min before use to allow it to cool to room temperature.
6. The selection of the objective lens depends on the specimen under investigation. In general, the higher the numerical aperture of the objective, the better the resolution. Moreover, water immersion lenses provide better resolution at deeper optical section, because of decreased spherical aberration.
7. For work with estrogen receptors the use of phenol red-free medium is recommended, to minimize estrogen activity contributed by the medium. For work with all steroid receptors, it may be necessary to use a serum substitute, dextran-coated charcoal stripped serum or serum-free medium to minimize hormonal activity contributed by serum.
8. The concentrations of DEAE–dextran (200–500 μg/mL), chloroquine (30–100 μ*M*), and expression plasmids (0.1–5 μg/mL) in the transfection solution, and the length of incubation (2–6 h), should be optimized for each cell line used. In some cell lines, it is preferable to incubate with chloroquine in serum free medium for 2 h following the DEAE–dextran transfection step. It is even more important to optimize the length of the shock step and to ensure that it is uniform from dish to dish. For a 6-min shock, a group of 12–20 dishes at a time can be taken through **Subheading 3.2.2.**, **steps 13–17**.

9. Although the flavonoids and phenol red in culture medium may autofluoresce, to a limited extent, the signal from GFP is usually strong and this background is slight. Autofluorescence is more of a problem when using excitation at the near ultraviolet wavelengths used for excitation of wild type GFP or BFP. If necessary, autofluorescence can be minimized by using phenol red-free medium or glucose-supplemented balanced salts solution for the brief scanning period. Cells can then be returned to culture medium if additional scanning is planned.
10. The use of higher-magnification laser-scanning (40–×100 objectives) may allow visualization of domains occupied by GFP-tagged receptors. For example, ligand-bound GFP–GR, GFP–PR, and GFP–ER are often observed to be concentrated in subnuclear clusters or speckles that may correspond to particular structures or functional domains. Colocalization experiments can be initiated to determine whether these correspond to subnuclear domains occupied by other proteins.
11. The issue of nuclear vs cytoplasmic localization can be obscured by a strong cytoplasmic signal if a standard fluorescence microscope is used. By optically sectioning a cell with the confocal instrument, it is possible to differentiate between GFP–receptor localized within the nucleus and GFP–receptor concentrated in the perinuclear cytoplasm.
12. The outcomes of intracellular trafficking kinetic experiments will be sensitive to temperature. The use of a temperature-controlled microscope stage and having the cell culture incubator located nearby can obviate this difficulty.

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14

Knockout Mice and Steroid Receptor Research

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1. Introduction

The development of techniques that allow defined alterations of the mammalian genome have dramatically increased the possibilities for elucidating functions of specific genes in the context of a whole organism. Although some of these techniques have been applied to several mammalian species, it is primarily the mouse that is the subject for these types of experiments. The first technique developed in this field was transgene technology, involving the incorporation of new copies of a gene into the genome of the host organism, and was successfully performed for the first time in 1980 ***(1,2)***. The other technique used to alter the mouse genome is referred to as "gene-targeting technology," or more popularly, "knockout technology." This technology involves homologous recombination in embryonic stem cells (ES cells) and was developed during the same decade ***(3–15)***. Gene targeting in ES cells has allowed scientists to perform studies of gene function in a way that has never been possible before. This review focuses on knockout technology, but also includes some background about transgenesis, because of its relevance for conditional knockouts. For more detailed information regarding establishment of transgenic mice, *see* **ref. *16***.

1.1. Principle of Transgene Technology

The mammalian genome is, to some limited extent, permissive to the introduction of new DNA. Transgene technology relies on the random introduction of genetic material into the genome, and in many cases this results in a successful integration of a gene of interest. The integrated gene, also referred to as the transgene, could be any gene that is of interest to an investigator; it could be

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derived either from the host genome or from a completely different genome, including one of viral origin.

The transgene is introduced into the genome via a constructed vector, a so-called "transgenic construct," which usually also includes regulatory sequences (promoter and/or enhancer) in order to control the expression of the transgene. The promoter/enhancer is chosen to direct either a ubiquitous or a tissue-specific expression pattern of the transgene, depending on the purpose of the experiment. In order to establish a transgenic animal, the transgenic construct is microinjected into fertilized eggs, which are then implanted into pseudopregnant foster mothers. Some of the progeny will harbor the transgene in their genomes, and these are subsequently tested to assess transgene expression.

Characteristic for transgenes is the concatemerization of the injected DNA, often resulting in the presence of many copies of the gene of interest. The number of copies of a mouse transgene affects its expression level, such that higher copy numbers sometimes result in lower expression, presumably because of the formation of a less accessible chromatin structure in multimeric arrays ***(17)***. In that study, a strategy based on the Cre/loxP recombination system was used to reduce the number of transgene copies to one, and this method can be used, in general, if there is a concern about the expression levels in a transgene experiment.

Another factor of importance for the expression level of the transgene is the site of integration in the genome. Sequences surrounding the integration site of the transgene often affect not only the level of its expression, but also the spatiotemporal pattern of expression. The latter sometimes occurs irrespective of the specific promoter/enhancer included in the transgenic vector, so that an intended tissue-specific expression is not achieved. It is therefore necessary to perform an expression screening of a large number of transgenic animals, each of which has the transgene integrated at a different site. Following the screening process, the animal expressing the transgene correctly is then selected to establish a new, transgenic line.

1.2. Principle of Gene-Targeting Technology

Gene targeting in the mouse allows genetic alterations that can be exactly defined by an investigator. This technology relies on the ability of mammalian cells to undergo homologous recombination ***(18)*** along with the possibility to culture and propagate ES cells in vitro ***(4,19)***. Knockout technology has so far only been utilized in the mouse, but it could in principle be used for other mammalian species as well. By usage of homologous recombination, a DNA sequence is replaced by a partially different sequence, thus creating alleles that harbor mutations, ranging from specific point mutations to large deletions. As mentioned, gene targeting allows for specificity in terms of the location within

the genome where the alteration will take place, and this is achieved via the homology between the introduced DNA and the target sequence.

Although there exist several different strategies describing methods for the introduction of genetic changes in a locus by homologous recombination, the most commonly used is termed "replacement" ***(18,20)***. Homologous recombination takes place spontaneously in mammalian cells, although it occurs at a low frequency. The possibility of culturing ES cells is crucial because these pluripotent cells have the potential to give rise to any cell type in the developing embryo. If kept under proper conditions, ES cells have the ability to proliferate a large number of times in vitro while still maintaining their pluripotent stem cell characteristics. Thus, by genetically manipulating a single ES cell by homologous recombination, an entire animal harboring a desired mutation can be generated.

The first step in performing a gene targeting experiment in the mouse is to create a targeting vector, also called a knockout vector. This involves the isolation of a suitable genomic fragment of the gene of interest from a genomic library. The library must be made from DNA isolated from the same mouse strain from which the ES cells were originally isolated, which will maximize the homology between the genomic DNA in the construct and the target DNA in the ES cell, which is important for the frequency of homologous recombination. The genomic fragment to be included in the targeting vector should be many kilobases (kb) in length, preferentially more than 8 kb, and it must contain the part of the gene where a mutation is to be introduced. For genes with only one exon, a so-called "null allele" can easily be created by simply deleting the whole exon. When it comes to multiexon genes, which contain long introns of many kb, a deletion of the whole gene may not be optimal because of the risk for undefined and unwanted deletions of unknown regulatory sequences or exons for other genes, and, in this case, it is recommended to only delete a vital exon within the gene of interest or to introduce premature stop codons. Thus, multiexon genes can be mutated in several different ways, depending on which exon is deleted.

The next step in the creation of the targeting vector is to introduce selection markers, which are genes coding for proteins conferring resistance or sensitivity to added antibiotics or nucleotide analogs, respectively. The selection markers are necessary for the enrichment of ES cells that have undergone homologous recombination. The most commonly used antibiotic resistance gene is the neomycin resistance gene (Neo^r), but also the hygromycin resistance gene (Hyg^r) has been used. The antibiotic resistance genes are used for positive selection because only ES cells that have the targeting vector introduced into their genomes will survive. The presence of the Neo^r gene in a

targeted locus can result in misregulation of adjacent genes in vivo ***(21)***. Furthermore, the *Neo*^{r} gene product is a phosphotransferase, which may explain reports on general effects on gene expression by the presence of *Neo*^{r} ***(22)***. For these reasons, it has become more common to use the Cre/loxP recombination to remove the positive selection marker (*Neo*^{r} or Hyg^r), after the selection procedure is finished and positive ES cell clones have been identified. Removal of the selection marker can be performed either in the ES cells or later in the mouse harboring the targeted allele.

Since random integration of the targeting vector is more frequent than homologous recombination, a second selection step, called "negative selection," has been devised to kill off cells with random integration. The most common negative selection gene is the *thymidine kinase* (*tk*) gene from the herpes simplex virus (HSV) ***(23)***. Thymidine kinase is able to phosphorylate and thereby activate certain nucleotide analogs, such as ganciclovir (GCV) and FIAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil), which after incorporation into DNA, will cause chain termination. The negative selection marker is placed in a flanking position in the targeting vector, and, because of its nonhomology with the target locus, this part of the vector will not be introduced into the genome upon homologous recombination. In contrast, if the targeting vector is introduced into the genome by random integration, the *tk* gene will in many cases also become integrated. As a result of the presence of the *tk* gene in ES cells with random integration, it is possible to select against these cells, by addition of suitable nucleoside analogs in the ES medium. An alternative to the *tk* gene as a negative selection marker is the diphtheria toxin-A gene ***(24)***. The presence of this gene in ES cells with random integration of the targeting construct will directly kill the cells without any additives.

Finally, when the targeting vector is completed, it is introduced (transfected) into ES cells by electroporation. During the first 24 h after transfection, the ES cells are grown without selection to allow initiation of resistance gene expression. Thereafter, the appropriate antibiotic is added to select for ES cells that have incorporated the selection marker into their genomes. Some days later, if the *tk* gene is used for negative selection, a nucleoside analog is added to the medium in order to select against random integration of the targeting vector. Despite double selection, only a fraction of the surviving ES cell clones will usually have undergone homologous recombination. Thus, in order to identify clones harboring the desired mutation, all surviving clones of ES cells must be analyzed, usually by Southern blot.

In the next step, ES cells from a correct clone are injected into mouse host preimplantation embryos at the blastocyst stage, which are then implanted into pseudopregnant foster mothers. The litters from these females will contain

some pups that are chimeric, i.e., they are a mix of cells from the host embryo and from the injected ES cells. Chimeric animals, mostly derived from the ES cells, are desirable since this would increase the probability that the chimera would be able to transmit the mutation through the germline to the next generation.

To enable assessment of the degree of chimerism, the ES cells and the host blastocyst are usually derived from strains with different coat colors. The chimeric animals will therefore have a mix of two coat colors, and chimeras with a high percentage of ES-cell-derived coat color will be selected for continued breeding. The strains most commonly used are the agouti colored strain 129 for ES cells and the black strain C57BL/6 for the host embryos. Male chimeric animals are more useful for breeding purposes, so most ES cell lines in use have an XY karyotype. Since the agouti color is dominant over black, crossings between a chimeric male and a C57BL/6 female would result in agouti-colored offspring if the sperm from the chimera was derived from ES cells. 50% of the agouti offspring should be heterozygous for the introduced mutation, unless the mutation has a deleterious effect as a single copy. If black pups are obtained, these are of no interest since they will be derived from sperm originating from the host cells in the blastocyst, thus never harboring the mutation.

In order to get animals that are homozygous for the mutation, heterozygous animals are intercrossed. The percentage of the offspring being homozygotes will depend on whether or not the mutation has any negative effect on development: 25% in case of no negative effect and 0% if there is a strong negative effect. All types of abnormalities in the animals being heterozygous or homozygous for the mutation will give information about where and when the targeted gene is of importance in the mouse.

1.3. Conditional Knockouts

In order to circumvent some of the limitations posed in knockout technology, a sophisticated method for studying gene function has been developed. Known as "conditional knockout technology," this strategy allows tissue- and/or time-specificity, in terms of where and when a gene of interest is to be knocked out. Thus, by performing gene knockout experiments in specific tissues and/or at defined time-points during the life of a mouse, it is potentially possible to gain more detailed information from a conditional knockout compared to a global knockout. Conditional knockout technology is of particular interest for the study of genes whose products have either direct or indirect systemic effects in the organism, e.g., steroid receptors. If the gene of interest is expressed both in the brain and in peripheral tissues, for example, it may regulate the expression of circulating factors from the brain, thereby affecting other tissues where the gene is also expressed. In this case, it would be impos-

sible to separate indirect systemic effects vs direct effects on a peripheral tissue in the global knockout of the gene. However, by generating mice with a conditional knockout limited to the peripheral tissue, studies of the specific function of the gene in that particular tissue would be allowed. Another example, in which a conditional knockout would offer new possibilities, is for genes in which global knockouts result in early embryonic lethality and when studies of gene function at later stages are impossible. By designing a conditional knockout that can be induced at a developmental stage, when the gene is no longer absolutely necessary to the embryo, further development of the embryo and subsequent studies of gene function would be allowed. Another strategy to circumvent embryonic lethality is to restrict the knockout to a particular tissue of interest, within the parameter that the gene is not crucial for the viability of the embryo in that particular tissue.

The conditional knockout technology has mostly been based on a recombination system derived from bacteriophage P1, called "Cre/loxP" ***(25–27)***. Cre is a recombinase and acts via its recognition site loxP. If a DNA sequence is flanked by loxP sites that are placed as direct repeats, the Cre recombinase will delete the whole sequence between the loxP sites, leaving one loxP site behind. This feature is used to delete, for instance, an important exon in a gene. Two mouse strains need to be generated in order to make a conditional knockout that is based on the Cre/loxP system. One strain will harbor a conditional allele of the gene of interest. The conditional allele is created by "floxing," that is, flanking with loxP sites, an important exon, then introducing this allele into the genome by homologous recombination. The floxing must be performed in such a way that the gene is left intact, meaning that the conditional allele is completely normal, as long as there is no Cre present. The second strain that needs to be established is a Cre transgenic line that expresses the Cre recombinase in a desired manner, e.g., only in a particular tissue. By crossing the strain harboring the conditional allele with the strain containing the Cre transgene, it is possible to generate offspring with tissue- and/or time-restricted knockouts of the gene of interest ***(28,29)***.

As mentioned earlier, the Cre/loxP recombination system is also used to remove the *Neo*^{r} selection marker after it has fulfilled its task for selection of ES cells. This is performed by using a floxed *Neo*^{r} gene in the targeting vector, and by expressing Cre after introduction of a transient Cre expression vector into a positive ES clone. A safer method for deleting the *Neo*^{r} gene, which does not involve manipulation at the ES cell level, is to cross heterozygous animals with a Cre-expressing transgenic mouse line ***(30)***. Those pups that are heterozygous in the offspring will harbor a targeted allele without the selection marker. Recently, a new concept for the removal of the selection marker was worked out ***(31)***. A self-excision cassette was devised, which removes the selection marker automatically in the chimeric male testis. Thus, the *Neo*^{r} gene

will be deleted from the beginning in the heterozygous offspring of these chimeras. The cassette is equipped with flanking loxP sites with the *Neo*^{r} gene and the Cre gene, driven by a testis-specific promoter, in between. As soon as the testis-specific promoter becomes active Cre expression starts, resulting in the removal of the whole cassette, including the *Neo*^{r} gene.

In order to regulate the knockout event in time, several strategies have been developed. The most promising method exploits the tetracyclin regulatory system together with the Cre/loxP recombination system ***(32)***. Using this recently described strategy, the expression of the Cre recombinase can be regulated spatially by a tissue-specific promoter and temporally, to any time-point of interest by doxycyline (a tetracycline analog).

Besides Cre/loxP, there is another recombination system that has been used in mice, namely, FLP/FRT ***(33)***, which is derived from yeast ***(34)*****,** and works according to the same principle as Cre/loxP, but has so far been used less, because of its lower efficiency in the mouse. This will probably change since recent improvements of the FLP recombinase ***(35)*** have made it possible to generate transgenic FLP mice ***(36)*** that are as good as previously described Cre mice in terms of recombination efficiency. The availability of two efficient and independent recombination systems in mice will undoubtedly allow more advanced studies in the future.

2. Materials

2.1. Cloning and Preparation of Gene-Targeting Vector

2.1.1. Cloning of Targeting Vector

1. Mouse strain 129 genomic library (*see* **Note 1**) (if the ES cells are derived from this strain).
2. Vectors with selection markers (*Neo*^{r} and *tk*, respectively) (*see* **Note 2**).
3. Vector DNA purification system, e.g., the Qiagen plasmid purification system.

2.1.2. Preparation of Targeting Vector for Electroporation of ES Cells

1. Restriction enzyme digestions are performed with fresh high quality enzymes to ensure complete linearization of the vector.
2. Purification of linearized DNA: phenol–chloroform–isomaylalcohol (PCIA) (25:24:1, vol); chloroform–isoamylalcohol (CIA) (24:1, vol); 3 *M* Na acetate, pH 5.2; 99.5% ethanol; 70% ethanol. The authors usually use ready-to-use neutral phenol (pH 8.0) (Life Technologies Gibco).

2.2. Culturing of ES Cells

1. There are a number of different lines of ES cells established in several laboratories. The authors have been successfully using two different ES cell lines called R1 ***(37)*** and GSI-1. The latter line is commercially available from Incyte Genomics. Both are established from the mouse strain 129.

2. ES cells are cultured in ES media, which is composed of Dulbecco's modified Eagle's medium (K-DMEM) (high glucose with Na pyruvate), supplemented with heat inactivated (+56°C for 30 min) fetal bovine serum (FBS), ES qualified) (*see* **Note 3**), to a final concentration of 15%; L-glutamine at 2 m*M* final; HEPES at 10 m*M* final; β-mercaptoethanol at 0.1 m*M* final; nonessential amino acids MEM at 0.1 m*M* final; gentamicin at 10 μg/mL final; leukemia inhibitory factor (LIF) (*see* **Note 4**) at 10^3 U/mL final concentration. All components are from Life Technologies Gibco.
3. Trypsin solution: 0.05% trypsin/1 m*M* ethylenediamine tetraacetic acid (EDTA) (Life Technologies Gibco).
4. 2X Freezing media (volume %): 60% ES medium, 20% FBS, and 20% dimethyl sulfoxide (DMSO) (Sigma).
5. 1.25X Freezing media (volume %): 75% ES medium, 12.5% FBS, and 12.5% DMSO.

2.3. Culturing of Mouse Embryo Fibroblasts

1. Mouse embryo fibroblasts (MEFs) are cultured in a medium composed of DMEM (high glucose without Na pyruvate), supplemented with heat inactivated (+56°C for 30 min) FBS (*see* **Note 3**) to a final concentration of 10%; Na pyruvate at 1 m*M* final; L-glutamine at 2 m*M* final; nonessential amino acids MEM at 0.1 m*M* final; gentamicin at 10 μg/mL final concentration. All components from Life Technologies (Gibco).
2. Gelatin solution: 0.5 g gelatin (300 Bloom; Type A; tissue culture grade, Sigma) is autoclaved in 500 mL sterile MilliQ water then stored at room temperature.
3. Mitomycin stock solution (1 mg/mL, 100X): One vial (2 mg, Sigma) is dissolved in 2 mL phosphate-buffered saline (PBS), sterile-filtered, and stored as 250-μL aliquots at –20°C.
4. Trypsin solution: 0.05% trypsin/1 m*M* EDTA (Life Technologies Gibco).
5. 2X Freezing media (volume %): 60% ES medium, 20% FBS, and 20% DMSO.
6. Mycoplasma detection: The authors use the Mycoplasma Plus PCR Primer Set from Stratagene.

2.4. Electroporation of ES Cells

1. Electroporation is performed in transfection buffer, which is 20 m*M* HEPES (pH 7.05), 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* D-glucose.
2. For electroporation: Gene Pulser (Bio-Rad).

2.5. Selection of Electroporated ES Cells

1. G418 stock solution: 10 mg/mL (based on the active component) in PBS (Life Technologies Gibco) sterilized by filtration and stored at +4°C (can be stored at +4°C for several months). G418 (Geneticin, Life Technologies Gibco).
2. GCV (*see* **Note 5**) stock solution: 1 ampule Cymevene™ (Roche) is dissolved in 10 mL sterile tissue culture grade water (Life Technologies Gibco), and gives

0.20 *M* conc. 1 mL of the 0.20 *M* solution is diluted 100× in sterile PBS to 2.0 m*M*. The 2.0 m*M* solution is a 1000X stock and is stored in 1-mL aliquots at –70°C.

2.6. Picking and Freezing of Surviving ES Clones

1. Picking medium is K-DMEM with 10 m*M* HEPES, final concentration.
2. 1.25X Freezing medium (volume %): 75% ES medium, 12.5% FBS (ES-qualified) (*see* **Note 3**), 12.5% DMSO.

2.7. Screening of Selected ES Clones by Southern Blot

2.7.1. Preparation of Genomic DNA from ES Cells

1. Lysis buffer I: 100 m*M* Tris-HCl, pH 8.5, 200 m*M* NaCl, 5 m*M* EDTA, 0.2% sodium dodecyl sulfate, 250 μg/mL proteinase K (added fresh). A stock of proteinase K (Roche) is 20 mg/mL in sterile water, stored at –20°C.
2. Extractions and precipitation: PCIA (25:24:1, vol:vol:vol), CIA (24:1, vol:vol); isopropanol; 70% ethanol; TE buffer (10 m*M* Tris-HCl, pH 8.0/0.1 m*M* EDTA).

2.7.2. Preparation of Genomic DNA from ES Cells (Alternate Protocol)

1. Lysis buffer II: 20 m*M* Tris-HCl, pH 7.6, 100 m*M* NaCl, 10 m*M* EDTA, 0.5% SDS, 100 μg/mL proteinase K (added fresh).
2. Saturated NaCl solution (>6 *M*).
3. 99.5% Ethanol; 70% ethanol; TE buffer (10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA).

2.7.3. Restriction Digestions of ES DNA and Southern Blot

The genomic DNA is digested in an enzymatic reaction containing the following components and final concentrations: 1X restriction enzyme buffer; 1 m*M* spermidine; 100 μg/mL bovine serum albumin, 50 μg/mL RNase A, 40–50 U restriction enzyme (high concentration stock, i.e., 40–50 U/μL).

2.8. Karyotype Analysis

1. Colcemid (Life Technologies Gibco).
2. 75 m*M* KCl: KaryoMax KCl (Life Technologies Gibco).
3. Freshly prepared fixative: methanol:acetic acid (3:1).
4. Giemsa stain (KaryoMax Giemsa Stain, Life Technologies Gibco).

2.9. Blastocyst Preparation, Injection, and Implantation

For the exact procedures and material requirements of these manipulations, *see* **refs. *38*** and ***39***.

1. M2 medium: 95 m*M* NaCl, 4.8 m*M* KCl, 1.19 m*M* KH_2PO_4, 1.19 m*M* $MgSO_4$, 23 m*M* lactate, 0.33 m*M* pyruvate, 5.6 m*M* glucose, 4 m*M* $NaHCO_3$, 1.71 m*M*

$CaCl_2$, 21 m*M* HEPES, pH 7.4., 4 g/L bovine serum albumin, 100U/mL penicillin, 5.0 μg/mL streptomycin-SO_4, 0.001% phenol red. The pH of HEPES (Ultrapure grade, Calbiochem) is adjusted to 7.4 with 5 *N* NaOH.

2. KSOM medium: 95.0 m*M* NaCl, 2.5 m*M* KCl, 0.35 m*M* KH_2PO_4, 0.20 m*M* $MgSO_4$, 10.0 m*M* lactate, 0.20 m*M* pyruvate, 0.20 m*M* glucose, 1.00 m*M* glutamine, 1 g/L BSA, 0.01 m*M* Na_3-EDTA, 25.0 m*M* $NaHCO_3$, 1.71 m*M* $CaCl_2$, 100U/mL penicillin, 5.0 μg/mL streptomycin-SO_4, 1X MEM essential amino acids (Life Technologies Gibco), 1X MEM nonessential amino acids (Life Technologies Gibco).
3. Blastocyst injection media (BIM): K-DMEM, 10% FBS (ES qualified), 10 m*M* HEPES.

3. Methods

3.1. Construction and Preparation of Gene-Targeting Vector

3.1.1. Construction of Targeting Vector

The order in which the cloning steps described below are performed is not important. What determines the order is whether unique sites are available for cloning, and which way will offer the most convenient strategy. A complete cloning plan is advisable for the whole project before any cloning work is started. This plan should also include the strategy for how to screen ES cell clones for homologous recombination. It is important to make sure that there is a unique restriction enzyme site available in the completed targeting vector, which can be used for linearization before electroporation of ES cells. This site must be located in a flanking position, either between the genomic sequence and the cloning vector or between the *tk* gene and the cloning vector.

The method for construction of a targeting vector used to establish mice harboring conditional alleles is described elsewhere ***(40)***, the reader is therefore referred to that manual for a description of flox vectors. Principles for the construction of a regular knockout vector are as follows:

1. A phage λ clone, a P1 clone, or a BAC clone, harboring a genomic fragment containing the gene of interest, is isolated from a genomic mouse library (*see* **Note 1**). As mentioned in **Subheading 1.2.**, the DNA needs to be isogenic with the ES cells.
2. The genomic fragment is digested with a panel of suitable restriction endonucleases in order to obtain a rough restriction site map. Using radioactive probes from one or several exons of the gene helps to more easily identify the different fragments in a Southern blot after restriction digestions.
3. A suitable smaller fragment is subcloned into a cloning vector (*see* **Note 6**), pBluescript (Stratagene), is often used. This smaller genomic fragment will be the basis for the targeting vector. The size of this fragment should preferably be >8 kb. A smaller fragment to be used as a flanking probe for detection of homologous recombination should be subcloned, either directly from the phage

λ clone or the P1 clone, or, in turn, be subcloned from the first subcloned genomic fragment. The flanking probe must not have any sequences in common with the genomic fragment included in the targeting vector, but should represent a sequence immediately 5' or 3' of this fragment.

4. The optimal situation is placing the insertion site for the positive selection marker (*see* **Note 6**) roughly in the middle of the genomic fragment. Neither of the homologous arms on each side of the nonhomologous selection marker gene should be shorter than 2 kb. There is a direct correlation between the size of the homologous genomic DNA and the frequency of homologous recombination. The frequency increases up to approx 14 kb ***(41)***. For larger fragments, shearing of the DNA in the electroporation process prevents further increases in frequency. It should be mentioned that the frequency of homologous recombination differs greatly between different loci in the genome. For some easily recombined loci, short fragment in the targeting vector may therefore be enough to obtain a decent frequency, but this may not be the case in some other loci. If possible, the desired mutation, for instance, a deletion could be introduced at the same time as the selection marker is added to the targeting vector.
5. The HSV-*tk* (*see* **Note 2**) gene is inserted in a flanking position in relation to the genomic DNA.
6. When the targeting vector has been completed, one must ensure that everything is correctly arranged. This is done by digesting the vector with a number of different restriction enzymes that should yield the expected fragment sizes. Polymerase chain reaction (PCR) and sequencing could also be used when this is suitable.
7. The purity of the targeting vector DNA preparation is important for the outcome of the ES cell transfection. For this reason, a protocol resulting in high DNA quality and purity should be used; the authors usually use the solid phase based purification system from Qiagen.

3.1.2. Preparation of Targeting Vector for Electroporation of ES Cells

1. The targeting vector is linearized with a suitable restriction enzyme.
2. The enzyme is heat-inactivated (if possible).
3. One extraction with PCIA and one with CIA are performed.
4. The DNA is precipitated at room temperature with one-tenth vol 3 *M* NaOAc and 2.5 vol 99.5% ethanol. Centrifugation at room temperature for 10 min at 20,000*g*.
5. The pelleted DNA is washed twice at room temperature in 70% ethanol.
6. Finally, the linearized and purified targeting vector is briefly dried under sterile conditions, then dissolved in sterile TE to obtain a DNA concentration of approx 1 μg/μL.

3.2. Preparation and Culturing of Feeder Cells

3.2.1. Preparation of MEFs from Embryos

Embryos 13–14 d old (E13.5–E14.5) from a transgenic mouse strain harboring the *Neo*^{r} gene (if this selection marker is used for positive selection of the

transfected ES cells [*see* **Note 7**]) are collected, and MEFs are prepared according to the following procedure:

1. The pregnant female is killed by cervical dislocation or with CO_2, and the abdomen is cleaned off with 70% ethanol. The female is opened up, and the uterus containing the embryos is aseptically transferred to a 50-mL tube with 20 mL sterile PBS, swirled around, then transferred with sterile forceps to a new tube to repeat the washing procedure.
2. The uterus is transferred to a Petri dish (non-tissue culture dish) with 20 mL PBS. All embryos are dissected out and washed twice as above in 50-mL tubes with 20 mL PBS. The embryos are then transferred to a Petri dish with PBS, and freed from amniotic membranes and placentas. The washing procedure is repeated. After transfer to a new Petri dish, the embryos are decapitated, and the heart and liver are gently squeezed out. The embryos are washed once and transferred to a dry Petri dish. Seven embryos per dish are then minced thoroughly with sharp and sterile scalpels.
3. 2 mL Warm (+37°C) trypsin solution (0.05% trypsin/1 m*M* EDTA) per embryo is added. The embryos are resuspended several times with a 10 mL pipet, then incubated at +37°C for 15 min.
4. The suspension is passed several times through a syringe fitted with a 18-gage needle. An equal volume of warm trypsin solution is added and another incubation is performed as above.
5. Transfer the suspension to a 50-mL tube and let undigested tissue debris settle for 2 min, then transfer the supernatant to a new 50-mL tube.
6. An equal volume of MEF medium is added, and the cells are counted. The yield is usually $1–2 \times 10^7$ cells/embryo.
7. The cells are spun for 5 min at 270*g*, then resuspended at 1×10^6 cells/mL in MEF medium.
8. 10 mL MEF suspension is then seeded per gelatinized (*see* **Subheading 3.2.2.**) 100-mm culture dish. After 24 h in culture (+37°C, 7.5% CO_2/95% humidity), the media is changed to remove those cells that have not attached to the dish (about 50% of the seeded cells). The dishes should be confluent after 2–3 d.
9. The MEFs are expanded from one 100-mm dish to one 150-mm dish as follows: The medium is aspirated and the dishes are washed once with warm (+37°C) PBS. Warm trypsin solution is added, and the dishes are incubated at room temperature in the sterile hood until the cells detach. 5 mL MEF media is added, and the cells are transferred to a 50-mL tube and centrifuged as previously described. The cells are resuspended in 25 mL MEF media and seeded onto 150-mm dishes.
10. The MEFs are harvested from the 150-mm plates 2–3 d later. The cells are counted, washed in MEF media, resuspended at 5×10^6 cells/mL in cold MEF media, then kept on ice. 1 mL Cell suspension is set aside for continued culturing, according to **Subheading 3.2.3.** An equal volume of cold 2X freezing media is added to the remaining cells. The cell suspension (now 2.5×10^6 cells/mL) is

divided on ice into 1 mL aliquots in cryogenic vials and frozen at –70°C, optimally, by lowering the temperature 1°C/min (*see* **Note 8**).

11. The next day the cells are transferred to liquid nitrogen for long-term storage.

3.2.2. Preparation of Gelatinized Culture Dishes

To gelatinize 100 mm culture dishes, 5 mL sterile 0.1% gelatin in PBS is added per dish, swirled around, and followed by an incubation at +37°C for 20–30 min. The gelatin solution is removed by aspiration. The dishes can then be used immediately, or stored at +4°C for up to 2 wk, but freshly prepared dishes are preferred.

3.2.3. Antibiotic Resistance Test and Mycoplasma *Screening*

19 mL of MEF media is added to the saved 1-mL cell suspension from **Subheading 3.2.1., step 10** and the cells are seeded onto two 100-mm cell culture dishes. These dishes are used to test for antibiotic resistance and *Mycoplasma* infection (by PCR), respectively. For the antibiotic test, the cells are grown to confluence, trypsinized, then treated to inhibit mitosis (*see* **Subheading 3.2.4.1.** or **3.2.4.2.**). The MEFs are seeded onto a 100-mm dish, then incubated for at least 10 d in MEF media supplemented with 300 μg/mL G418.

3.2.4. Mitotic Inactivation of MEF Cells

Frozen MEF feeder cells are quickly thawed in a +37°C water bath. When the last ice crystals have disappeared, the cells are transferred into a 15-mL tube with 9 mL ice-cold MEF medium. Followed by a centrifugation at +4°C for 5 min at 270*g* and aspiration of the supernatant, the cells are resuspended in 10 mL MEF medium. The washing procedure is repeated.

Cells are counted, then seeded out at 5×10^6 cells/100-mm culture dish (*see* **Note 9**). The MEFs are grown to confluence (3–4 d), then split 1:3 and grown for another 3–4 d until they are confluent again. To avoid further cell divisions: The cells are then treated in one or two ways, either by mitomycin C treatment or by γ-irradiation.

3.2.4.1. Mitomycin C Treatment of MEFs

1. Confluent cells are trypsinized and seeded in MEF media on new 100-mm dishes at 30,000 cells/cm^2.
2. The cells are incubated in MEF medium containing 10 μg/mL mitomycin C at +37°C for 2–6 h.
3. The MEFs are washed three times in PBS, trypsinized, counted, spun down in MEF medium, and then resuspended in MEF medium at 5×10^6 cells/mL.
4. The cells are seeded on 100 mm dishes at 50,000 cells/cm^2. The MEFs are allowed to attach overnight before being used as feeder cells for the ES cells. If the MEFs

are not used immediately, the medium should be changed every 3 d. The cells should be used within 10 d.

3.2.4.2. γ Irradiation of MEFs

1. Confluent cells are trypsinized, then resuspended in 7 mL cold MEF medium. The cells are kept on ice, counted, spun down, and resuspended at 2×10^6 cells/mL in cold MEF medium.
2. The cells are then irradiated at 3000 rad (30 gy) with a cesium irradiator, and seeded onto 100-mm dishes at 50,000 cells/cm^2. The MEFs are allowed to attach to the dishes overnight. MEF feeder cells for 24-well plates are seeded at 80,000 cells per well.

3.3. Culturing of Embryonic Stem Cells

In order to be successful in a gene targeting experiment, top-quality ES cells are of critical importance. To maintain their pluripotency, and to avoid differentiation, these cells need devoted attention on a daily basis. They usually need new medium every day and should never be allowed to grow too densely. MEF feeder cells and presence of LIF are absolute requirements to keep ES cells undifferentiated and healthy.

1. ES cells are thawed (*see* **Note 10**; **Subheading 3.2.4.**) and frozen (**Subheading 3.2.1., step 10**) according to the same procedure as described for MEFs, except that ES medium is used instead of MEF medium.
2. After thawing, the ES cells ($1–2 \times 10^6$ cells) (*see* **Note 11**) are seeded in ES medium onto 100-mm dishes with mitotically inactive MEF feeder cells, prepared as described in **Subheading 3.2.4.**
3. The medium is changed daily and the third day after thawing, the ES cells are split 1:2 and seeded onto two new 100 mm MEF dishes.
4. The following day the ES cells are transfected by electroporation according to the procedure described next.

3.4. Electroporation of ES Cells

Introduction of the targeting vector into the ES cells is performed by electroporation. The procedure for the preparation of the targeting vector before electroporation is described in **Subheading 3.1.2.** The purity of the targeting vector is of importance for the outcome of the transfection and should not be disregarded.

1. Two hours before electroporation, the medium is carefully aspirated and fresh, warm (+37°C) ES medium is added to the ES cells on two 100 mm dishes.
2. Immediately before electroporation, the ES cells are washed in PBS, trypsinized, and transferred to one separate tube from each of the two dishes. The cells are spun down at 270*g* for 5 min at room temperature and subsequently resuspended in 0.8 mL transfection buffer per tube.

3. 25 μg (at approx 1 μg/μL in TE) of DNA are added per tube at room temperature. The cells and the DNA is carefully mixed, then transferred to electroporation cuvets. Without any delay, the ES cells are electroporated at 0.23 kV and 500 μF. The pulse duration should be about 7–8 ms.
4. Immediately after electroporation, the cell suspensions are transferred from the two cuvets to one 50-mL tube with 40 mL prewarmed (+37°C) ES medium.
5. The ES cells are subsequently seeded onto four MEF feeder dishes (10 mL suspension per dish), and incubated at +37°C/7.5% CO_2/95% humidity. The cells are then treated as described in the following subheadings.

3.5. Selection of Electroporated ES Cells

1. When the electroporated ES cells have been in culture for 24 h, the medium is carefully aspirated and fresh ES medium, supplemented with the appropriate antibiotic, is added. In parallel to the electroporated cells, a control dish with nonelectroporated ES cells with the same cell density should be set up. These cells are used to check that the antibiotic selection step is working.
2. The medium should be changed every day and the antibiotic should be present during the whole selection process.
3. Four days after electroporation, GCV (2 μ*M* final) is also included in the ES media to start negative selection. This selection step will stop growth of clones with random integration of the targeting construct.
4. Continue the daily changes of medium, now including both the antibiotic and Ganciclovir.
5. Eight days after electroporation, the cells on the control plate should have been completely killed off by G418.

3.6. Picking, Culturing, and Freezing of Selected ES Clones

1. If all control cells are dead, and if the surviving electroporated ES clones are big enough (at least 200 cells/clone), it is time to pick these clones. Usually, this is 8–9 d after electroporation.
2. Before picking the ES clones, the medium is aspirated and replaced with 10 mL serum-free ES medium.
3. The clones are picked under microscope at low magnification using a 20-μL micropipet set to 2 μL and fitted with filtered tips. The clones are placed in individual wells in a round-bottomed, 96-well plate containing 25 μL trypsin solution. When eight clones have been picked (one vertical row filled), the ES cells are incubated at +37°C for 5 min, then disaggregated by pipeting up and down 15–20 times with a 8-channel multipipet. Check the ES cells under microscope. If necessary, continue the incubation at +37°C until a single cell suspension has been obtained.
4. Immediately add 100 μL ES medium to stop the trypsin reaction. Carefully suspend the ES clones in the medium, then transfer them into two parallel 48-well plates (50 μL cell suspension per well) with MEF feeder cells and 150 μL ES medium with 300 μg/mL G418 per well. One 48-well plate is used for freezing

the ES cells, and the other is used for DNA extractions and screening for homologous recombination.

Optional: The MEF feeder cells can be omitted from the plate for DNA preparation. This is actually recommended if the MEF wild-type fragment detected in the Southern blot screening is of a different size than to the wild type fragment in the ES cell.

5. The medium is changed daily on the plate intended for freezing. The ES clones should be frozen well before they reach confluence.
6. To freeze the clones on the 48-well plates, two rows (16 wells) are processed at a time. The medium is first carefully aspirated and the cells are washed once in PBS. 100 µL trypsin solution is added to each well, followed by an incubation at +37°C for 5 min. The ES cells are resuspended with a pipet (check under microscope that a single cell suspension has been obtained), then transferred to a new 48-well plate on ice with 400 µL 1.25X freezing media per well. The cells are suspended by pipeting up and down several times. When all wells on the original 48-well plate have been processed, the new plate is frozen at –70°C, optimally, by lowering the temperature 1°C/min (*see* **Note 8**).

3.7. Screening of Selected ES Clones by Southern Blot

3.7.1. Preparation of Genomic DNA from ES Cells

The 48-well plate, with ES clones intended for DNA preparation and screening, should be grown to confluence, to obtain as much DNA as possible. It does not matter if these ES cells differentiate or not. The media is aspirated, and the cells are washed twice with PBS at room temperature. At this point, the cells can either be frozen (dry, after aspiration of all PBS) and kept at –20°C or processed immediately, as follows:

1. 500 µL of lysis buffer I (*see* **Subheading 2.7.1.**), including 250 µg/mL final concentration of proteinase K (added fresh), is added to each well. The cell lysates are then transferred to 1.5-mL microcentrifuge tubes and incubated at +55°C for at least 2 h (or overnight).
2. The samples are extracted with 1 vol of PCIA by shaking tubes by hand for 3–5 min. The tubes are then centrifuged for 5 min at 20,000*g* in a microcentrifuge. The supernatants are transferred to new tubes and extracted with an equal volume of CIA (tubes shaken for 10 s). Centrifugation as above.
3. The supernatants are transferred to new tubes and an equal volume of isopropanol is added. The tubes are mixed and the DNA is pelleted by centrifugation as in **step 2**. 1 mL of 70% ethanol is added to wash. Spin again, then dry pellets briefly.
4. The washed DNA precipitates are dissolved in 20 µL TE. To dissolve the DNA, the tubes are incubated at +55°C for 1 h and then immediately put on ice. Flicking the tubes facilitates the dissolving process. If necessary, a shorter incubation

at +55°C, followed by ice, is repeated until the DNA is completely dissolved. 10 µL of DNA is used for restriction enzyme digestions as described in **Subheading 3.7.3.**

3.7.2. Preparation of Genomic DNA from ES Cells (Alternate Protocol)

The authors have also used an alternative protocol for isolation of ES cell DNA. This results in more genomic DNA but requires one more step of expansion of the ES cell clones.

1. The ES cells intended for DNA preparation are expanded from 48-well plates to 12-well plates and grown to confluence.
2. The ES cells are washed twice in PBS, and 600 µL lysis buffer II is added (*see* **Subheading 2.7.2.**). The lysates are transferred to 1.5-mL microcentrifuge tubes, and then incubated overnight (or >2 h) at +55°C.
3. 300 mL Saturated NaCl is added and the mix is shaken for 3 min.
4. The samples are incubated on ice for 10 min and then centrifuged for 10 min at 20,000*g* at +4°C.
5. 650 µL of the supernatant is transferred to 2 mL microcentrifuge tubes and 2 vol of 99.5% ethanol is added. The tubes are mixed and the DNA precipitates are transferred to new tubes with 1 mL 70% ethanol to wash for 5 min. Only one sample at a time is processed according to this step in order to avoid prolonged ethanol precipitation periods.
6. The washed DNA precipitates are transferred to new tubes with 100 µL TE and dissolved as described in **Subheading 3.7.1, step 4.**

Both protocols described here for the preparation of genomic ES cell DNA are laborious. The authors have also used a third method, in which all purification steps are performed in 96-well plates ***(42)***. This method is considerably less time consuming, but results in smaller amounts of DNA.

3.7.3. Restriction Digestions of ES Cell DNA and Southern Blot

Half of the genomic ES cell DNA prepared as above (10 µL) is digested in a total volume of 25 µL in an enzyme reaction mix as described in **Subheading 2.7.3.** Digestions are performed at +37°C overnight. 5 µL 6X DNA loading buffer is added per tube, and the digested DNA samples are loaded on a 0.8% agarose gel. The following steps are then performed according to standard Southern blot procedures; in this laboratory, the authors usually use Amersham's Hybond-N membranes and the recommended protocol from this manufacturer. The fragment to be used as a flanking probe for detection of homologous recombination is released from the vector with the appropriate restriction enzyme(s), then purified twice on a gel. It is radioactively labeled to a high specific activity (>10^9 cpm/µg DNA) with random priming.

3.8. Expansion of Positive ES Clones

When positive clones have been identified, they are thawed and expanded. The procedure is as follows:

1. 24-Well plates with mitotically inactivated MEF cells are prepared.
2. Change from MEF medium to ES medium immediately before the ES cell clones are thawed.
3. The 48-well plates with frozen ES cell clones are quickly thawed at +37°C. About 400 μL of the medium is carefully removed from the wells of positive clones (the ES cells should be on the bottom). 400 μL Fresh and warm (+37°C) ES medium is added and the cells are resuspended. One clone is transferred to one well on a 24-well plate containing MEFs and 500 μL ES media.
4. After 3 d, the clones on the 24-well plates are split 1:6 onto new 24-well MEF plates. Four of the wells are frozen as stocks, one is for DNA preparation to confirm positive clones, and one is for karyotype analysis.

   Optional: The MEF feeder cells can be omitted from the plate for DNA preparation. This is actually recommended if the MEF wild-type fragment detected in the Southern blot screening is of a different size compared to the wild type fragment in the ES cell.

3.9. Karyotype Analysis of Positive ES Cell Clones

This procedure ensures that the ES clones subject to blastocyst injections have the correct chromosome numbers. Also, larger chromosome aberrations can be detected.

1. 0.5 mL ES medium containing 0.2 μg/mL colcemid is added to ES cells that are 60–80% confluent in a 48-well. Incubated 2–12 h. The cells will round up from the colcemid treatment.
2. The cells are trypsinized and resuspended in 400 μL ES medium, then transferred to a 1.5-mL microcentrifuge tube. There is no need to work under sterile conditions from now on.
3. The cells are centrifuged at 270*g* for 5 min. The supernatant is carefully removed and the cells are resuspended by flicking the tube.
4. 600 μL 75 m*M* KCl is added and the contents are gently mixed by inverting the tube. Incubated for 15 min at 37°C. **Note:** Cells become fragile at this point.
5. 25 μL Freshly prepared fixative [methanol:acetic acid (3:1)] is added. Mixed gently. Centrifuged for 5 min at 150*g* at room temperature.
6. 310 μL Supernatant is carefully removed. 310 μL fixative is added and the cells are resuspended gently. Centrifugation as in **step 5**.
7. 500 μL Supernatant is carefully removed and 400 μL fixative is added. The cells are resuspended gently, then centrifuged again as in **step 5**.
8. 450 μL Supernatant is carefully removed and 60 μL fixative is added. The cells are gently resuspended.
9. 35 μL Suspension is applied onto a microscope slide with a micropipetor as follows: From a distance of 25–30 cm, with careful aim allow 2–3 drops to

fall onto the center of a microscope slide. This action causes the fragile cells to burst, releasing the chromosomes. The slide is immediately run four to six times through the top of the flame from a Bunsen burner, until 80–90% dryness. **Note:** The slide should never become hot because this will destroy the chromosome architecture. This step is meant to quickly remove the fixative, thereby preventing the applied sample from spreading too much over the surface of the slide.

10. The slide is stained in Giemsa stain (1:10 dil) for 15–20 min, then briefly rinsed in deionized water. The slide is air-dried and a cover glass is then mounted onto the slide.
11. The slide is examined under the microscope. Scan for good metaphases using a ×10 objective. Switch to a ×100 oil-immersion objective to count chromosomes (40 chromosomes is the normal karyotype for the mouse). Also look for other chromosomal aberrations.

3.10. Blastocyst Injections

Injection of ES cells into blastocysts and the subsequent implantation of these blastocysts into pseudopregnant females requires a well-functioning animal facility and special equipment. For the exact procedures of these manipulations, *see* **refs. *38*** and ***39***, where the techniques involved are extensively described with both text and illustrations.

3.10.1. Preparing ES Cells for Blastocyst Injection

To prepare ES cells for blastocyst injection, the following steps are taken:

1. A positive ES clone is thawed (or proceed directly from **Subheading 3.8., step 3**) and seeded in ES medium (no G418) on 24-well plates with MEF feeder cells. The cells are seeded at different densities in different wells to ensure that the optimal conditions are reached in at least one well. The cell density should be neither too low or too high and the ES cells should never reach more than 70% confluence.
2. Ideally, the ES cells should be passaged once after thawing, then seeded onto 24-well plates again as described previously. For best results, the ES cells should be in logarithmic growth phase when injected into blastocysts.
3. The ES cells are grown until the individual clones contain 20–100 cells. The medium is changed 2–4 h before blastocyst injection. If all individual clones look good, the whole well can be trypsinized. Alternatively, individual and healthy-looking clones are picked, pooled, and trypsinized.
4. The cells are disaggregated by pipeting. Check under a microscope to see that a single cell suspension has been obtained.
5. The trypsin is immediately inactivated with 1 mL cold (+4°C) and freshly prepared BIM. The cell suspension is transferred to a 15 mL tube with 9 mL cold BIM.
6. The cells are centrifuged at 270*g* for 5 min at +4°C, then resuspended in 10 mL cold BIM.

7. **Step 5** is repeated twice and the pelleted cells are then finally resuspended in 200 μL cold BIM. The cells are kept on ice until blastocyst injection, which should be performed without any unnecessary delay.

3.10.2. Preparing Blastocysts for Injection of ES Cells

Blastocysts are prepared to be available when the ES cells are ready for injection. Briefly, this is performed as follows (*see* **refs. 38** and **39** for details):

1. Inbred C57Bl/6 mice are mated (*see* **Note 12**), and, in the morning, 4 d post-coitum (dpc), the females are sacrificed by cervical dislocation, and the uterine horns are dissected out. The blastocysts are flushed out with M2 medium.
2. When all blastocysts have been collected from one female, they are transferred with a mouth controlled pipet to KSOM media. The blastocysts are incubated in a tissue culture incubator at +37°C/5.5% CO_2/95% humidity for 1–2 h, then inspected under microscope. Only healthy-looking blastocysts are used for injections.
3. Injections are performed under a high-quality stereomicroscope, equipped with joy stick manipulators that are fitted with a holding pipet and an injection pipet. The blastocyst to be injected is kept in place with the holding pipet in KSOM medium. 10–20 ES cells are placed in the injection needle, then carefully injected into the blastocyst.
4. The injected blastocysts are kept in KSOM medium in the tissue culture incubator until implantation.

3.11. Implantation of Blastocysts

Foster mothers are prepared to be ready for implantation the day of blastocyst injection. These females will be able to receive blastocysts after being mated to sterile (vasectomized) males. Six to seven blastocysts are implanted per uterine horn.

3.12. Breeding of Mice

The likelihood that a chimeric male will transmit the introduced mutation through the germline to the next generation can be judged from the coat color. The more of the ES-cell-derived agouti color there is, the more likely it is that also a majority of the sperm originates from the manipulated ES cells (*see* **Note 13**). Good ES cell clones can give rise to chimeric males with an almost 100% agouti-colored coat.

The best chimeric males are selected for breedings with C57Bl/6 females. Tail biopsies are taken from the agouti colored offspring (*see* **Subheading 3.13.**) and the genomic DNA is screened for presence of the mutated allele. 50% of the agouti pups should be heterozygous, if there is no negative selection against the mutated allele. Heterozygotes are then intercrossed and the phenotype of the mutant offspring is analyzed. One important issue to consider

at this stage of the gene-targeting project is the genetic background. It has been demonstrated ***(43)*** that the genetic background can dramatically change the phenotypical effect of a knockout. It is therefore advisable to cross the heterozygotes with one or several inbred strains, then do backcrosses with the respective strain for at least 10 generations in order to obtain a more defined genetic background. The heterozygote animals obtained after 10 generations, of backcrosses are intercrossed within the respective line and mutant animals are then analyzed.

3.13. Tail Biopsies and Preparation of Genomic DNA

1. About 3–4 mm of the tail tip is cut from pups at 12–16 d of age. Biopsies are collected in 1.5-mL microcentrifuge tubes including 500 µL lysis buffer with freshly added proteinase K.
2. Incubate samples overnight at +55°C.
3. The next day, the tubes are vortexed briefly. Nondigested material (hair) is spun down for 10 min at 20,000*g* at room temperature.
4. Carefully pour the supernatants into 1.5 mL microcentrifuge tubes containing 500 µL of isopropanol. Mix gently until a visible precipitate appears, but do not mix more than necessary. Mixing too many times makes the DNA condense and hard to dissolve later. Process one sample at a time from this step to the next, to avoid excessive precipitation times in isopropanol, thereby minimizing coprecipiation of salts.
5. Transfer the precipitate with a micropipetor into a new tube with 1 mL 70% ethanol to wash.
6. Transfer the washed precipitate into a new tube with 250 µL 10 m*M* Tris/0.1 m*M* EDTA. Dissolve the DNA at +55°C, followed by ice. Mix until DNA is dissolved (avoid vortexing).
7. The DNA concentrations are measured spectrophotometrically.

3.14. Screening for Mutated Allele (Genotyping)

Determination of the genotype of pups can be done either with the same restriction enzyme digestion/Southern blotting procedure as was used to screen the ES clones for homologous recombination, or alternatively, a PCR-based procedure can be used.

1. For Southern blot screening, approx 10 µg is digested overnight with the appropriate restriction enzyme according to **Subheading 3.7.3.**
2. For PCR screening, an aliquot of the genomic DNA is diluted 1:10 in water and 1–5 µL is then used in a PCR reaction. To enable discrimination between heterozygotes and homozygous mutants in just one PCR step, it is beneficial to work out a strategy involving three primers in the same reaction. This should be designed to result in two products with different sizes for heterozygotes (if this is possible), one from the wild type allele and one from the mutated allele.

4. Notes

1. Genomic clones are usually isolated from phage λ genomic libraries (Stratagene), if this is performed in the laboratory. Alternatively, there are possibilities for obtaining specific genomic clones from P1 or BAC libraries on a commercial basis from Incyte Genomics. In this case, the investigator supplies PCR primers that are specific for the gene of interest, and the company will then return one or several P1 or BAC clones harboring specific genomic sequence.
2. The vector, pMC1neopolyA ***(10)***, is commercially available from Stratagene. There are floxed neo vectors described that can be used if the *Neo*^{r} gene is to be removed, e.g., pL2neo ***(40)***. The self-excision cassette described by M. Bunting et al. ***(31)***, called ACN, takes care of the *Neo*^{r} gene excision on its own in the male germ line, thus making this step easy. The HSV-*tk* vector was devised by Mansour et al. ***(23)***.
3. The quality of the FBS is very important. For culturing of ES cells, the authors only use specially tested and ES-cell-qualified FBS from Life Technologies Gibco. For MEFs, the authors use regular tissue grade FBS.
4. LIF is from Chemicon, 10^6 U/mL.
5. Ganciclovir is unstable in water and some investigators make fresh stock solutions every day. However, since the substance is classified as a cancer promoter, one compromise between daily handling and freshness is to make a stock that is stored at –70°C.
6. Some loci are prone to rearrangements, sometimes making it difficult to assemble a targeting vector in a plasmid. The stability of genomic DNA is much higher in bacteriophage λ vectors compared to plasmids, and a method to perform the whole targeting vector construction in a phage λ vector has recently been described ***(44)***.
7. Usually, the *Neo*^{r} gene is used as selection marker, but, if the *Hyg*^{r} gene is used, the MEFs must be resistant to hygromycin.
8. There are advanced devices, available commercially, to assure optimal freezing conditions. Nalgene offers products that suit the budgets of most laboratories. Alternatively, the cryogenic tubes with the cells can be wrapped in several layers of bubble plastic and styrofoam containers, in order to make the cells slowly attain the surrounding temperature.
9. MEFs are able to divide about 10 times after isolation from embryos. MEFs older than four passages should not be used as feeders for ES cells.
10. When cells are thawed, they must be kept cold until the DMSO in the freezing medium has been washed away. DMSO penetrates the cell membranes and creates holes that prevent growing ice crystals, which are formed during the freezing procedure, to burst the cell. At higher temperatures, DMSO may be too active and could cause excessive cell damage, unless the cells are kept on ice.
11. Counting of ES cells: Many of the cells present in the suspension are feeder cells, and these have to be subtracted from the total cell number. In order to do this, a parallel dish with MEFs is counted. This dish should have been prepared with feeder cells at the same time as the culture dish (the same batch) where the ES cells were grown to ensure a correct calculation of the MEFs.

12. Some investigators treat females with hormones in order to increase the number of blastocysts, but the authors have found that the quality of the blastocysts is better from nontreated females.
13. There are strain differences between 129 and C57Bl/6 mice that should be considered here. C57Bl/6 mice develop faster, which makes sperms that are derived from the embryo host cells develop faster than the ES-cell-derived sperms. Thus, it is common that the first litters from a young chimera only contain black pups, but litters born some weeks later contain agouti pups when the ES-cell-derived sperm is being produced in larger quantities. Good chimeras, however, usually never give rise to black pups and all pups from the first litter are usually agouti. If there are problems getting germline transmission from the obtained chimeric males, one rule of thumb is to keep on trying until 100 pups have been born. The quality of the chimeras is mostly dependent on the quality of the particular ES clone. It is therefore recommended that, if there are problems in obtaining agouti offspring, a second positive ES clone is soon thawed to establish new chimeric males.

5. Mouse Genetics and Knockouts on the World Wide Web

The amount of information on the mouse and its genetics is rapidly growing. The following websites represent useful sources:

http://www.nih.gov/science/models/mouse/ (Trans-NIH Mouse Initiative)
http://www.informatics.jax.org/ (Mouse Genome Informatics)
http://lena.jax.org/resources/documents/imr/ (Induced Mutant Resource, IMR)
http://www.mshri.on.ca/nagy/cre.htm (Cre transgenic database)
http://www.biomednet.com/db/mkmd (BioMedNet, Mouse Knockout & Mutation Database)
http://www.ki.se/kfc/meg/Index.html (Unit for Embryology and Genetics, Huddinge University Hospital and Karolinska Institutet)

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15

Yeast Two-Hybrid Screening for Proteins that Interact with Nuclear Hormone Receptors

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1. Introduction

The yeast two-hybrid system, originally developed by Fields and Song ***(1)***, is a sensitive genetic assay for the detection of protein–protein interactions. The system exploits the fact that eukaryotic transcriptional activators contain separable functional domains for DNA-binding (domain [DBD]) and transactivation (activation domain [AD]) ***(2)***. These domains cannot activate transcription when expressed as separate entities in yeast (either alone or together). However, they can function when joined noncovalently via protein–protein interactions. Thus, any pair of proteins that interact with each other may be used to bring separate DBDs and ADs together to reconstitute a functional transactivator. In a typical two-hybrid assay, one protein termed the "bait," is expressed as a fusion with a specific DBD; the other is fused to an AD. If the two proteins interact in a yeast nucleus, transcription of reporter genes containing DBD sites will be enhanced. Using this approach, known proteins can be assayed for interaction, mutant proteins that are unable to interact with a given protein can be isolated, and libraries of AD fusion proteins can be screened for those that interact with a protein of interest. The authors laboratory has used domains from various steroid and nonsteroid nuclear receptors (NRs) as bait sequences to identify several interacting proteins that may mediate their transcriptional effects ***(3–8)***.

Nuclear receptors represent a large family of sequence-specific transcription factors that are regulated in many cases by the binding to specific lipophilic ligands such as steroid and thyroid hormones, retinoids, and vitamin D ***(9,10)*** Like other transcription factors, NRs have a modular structure with distinct

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domains for DNA binding, dimerization, ligand binding, and transactivation ***(11,12)***. In recent years, it has become apparent that the function of NRs is mostly defined by their physical interactions with a number of cellular proteins ***(13–16)***. In the absence of ligand, steroid receptors are associated with a complex of heat shock proteins that prevent their interaction with DNA. Ligand binding induces a conformational change in the receptor that releases heat shock proteins, and permits the receptor to bind its cognate response element and interact with a variety of coactivator proteins ***(13)***. In contrast to the steroid receptors, the nonsteroid receptors are capable of binding to DNA even in the absence of ligand and repress basal transcription of target genes through interaction with corepressor proteins. The presence of ligand causes the release of corepressors and the recruitment of coactivators, whose function is to remodel chromatin structure and/or to stimulate (pre)initiation complex formation ***(14–16)***.

NR-interacting proteins have been identified using yeast two-hybrid screening, functional complementation studies, far Western blotting, and expression cloning. This chapter describes yeast two-hybrid protocols that have been used to isolate and characterize proteins that interact with the estrogen receptor (ER) and retinoid (RAR and RXR) receptors. These protocols should also be applicable for screens using bait sequences unrelated to NRs.

2. Materials

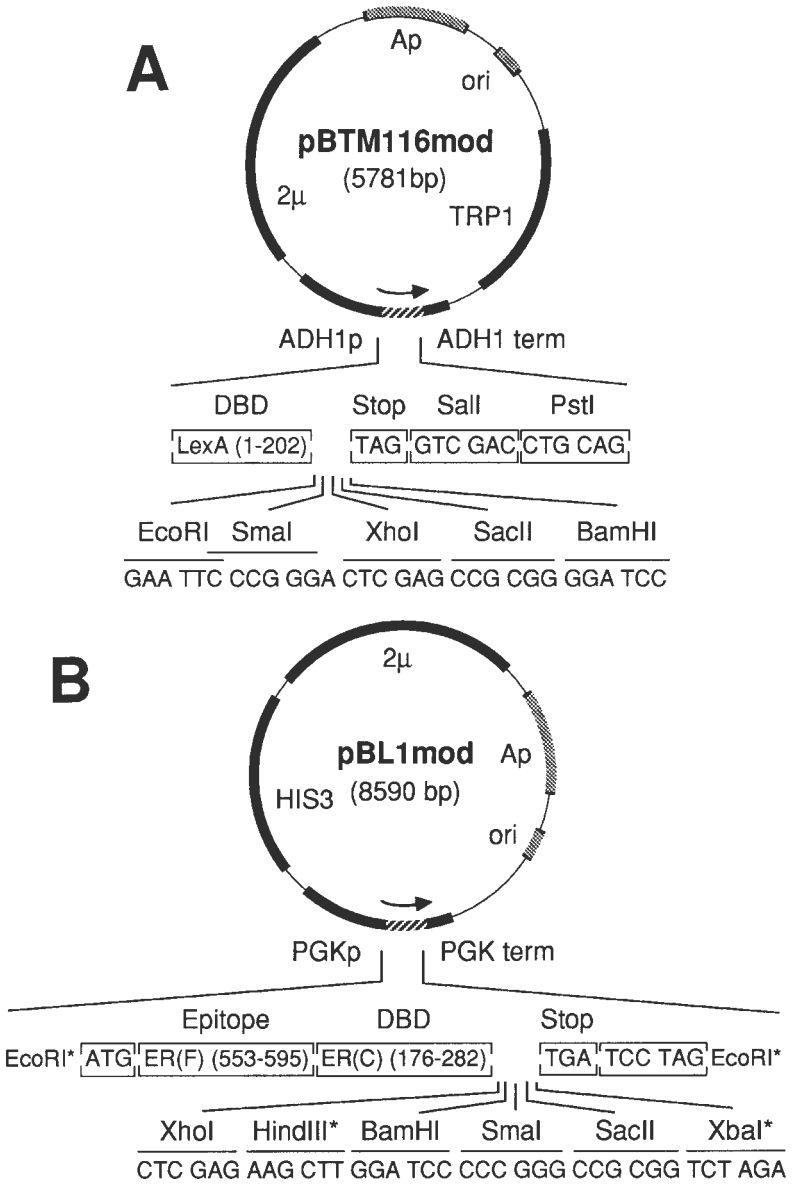
2.1. Plasmids

The yeast two-hybrid plasmids are shuttle vectors that typically contain sequences for replication, maintenance, and selection in *Escherichia coli* and *Saccharomyces cerevisiae* (**Figs. 1** and **2**).

2.1.1. DBD Vector

The most commonly used DBD vectors employ the DBD from either the yeast transcription factor GAL4 ***(1,17,18)*** or the bacterial repressor protein, LexA ***(19,20)***. An alternative system developed in this laboratory is based on the DBD of the human ERα ***(21)***. In these vectors, sequences encoding the DBD are followed by a multiple cloning site, in which the bait cDNA is introduced with the reading frame preserved. In most cases, the fusion protein is expressed from a strong constitutive promoter (alcohol dehydrogenase 1

Fig. 1. *(opposite page)* The DBD vectors, pBTM116mod **(A)** and pBL1mod **(B)**. The plasmid pBTM116mod is a derivative of pBTM116 ***(19)*** and uses the constitutive promoter from the yeast *ADH1* gene to express baits as fusions to the native bacterial repressor protein LexA. The plasmid pBL1mod is a derivative of pBL1 ***(21)*** and uses the constitutive promoter from the yeast *PGK* gene to express baits as fusions to a cas-



sette that includes an epitope tag from the human ERα (ER[F]; AA 553–595) and the ERα DBD (ER[C]; AA 176-282). Both plasmids also contain an *E. coli* origin of replication (*ori*), the ampicillin resistance gene (*Ap*), a yeast selectable marker gene (*TRP1* or *HIS3*) and a yeast origin of replication (2 μ). The polylinker sequences are shown as in-frame triplets. Restriction sites that are not unique are indicated by an asterisk.

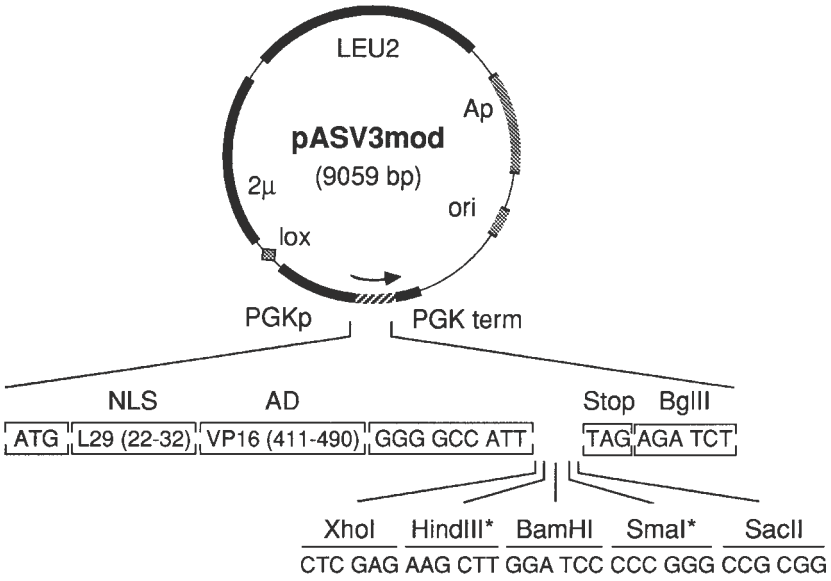


Fig. 2. The AD vector pASV3mod. The plasmid pASV3mod is a yeast–*E. coli* shuttle vector that directs the synthesis of AD fusion proteins under the control of the *PGK* promoter. The initiation codon, the nuclear localization signal (NLS) from the yeast ribosomal protein L29 (AA 22–32), the VP16 AD (AA 411–490), and the sequence of the polylinker (shown as in-frame triplets) are depicted. Restriction sites that are not unique are indicated by an asterisk. In addition to the sequences for replication and selection in yeast and in *E. coli*, pASV3mod contains a direct repetition of two lox sites flanking a *Not*I restriction site. The corresponding λ phage vector, λASV3, has been created to facilitate contruction of large VP16 AD-tagged cDNA libraries that can then be converted to a plasmid library by using the Cre-lox site-specific recombination system.

[*ADH1*], phosphoglycerate kinase [*PGK*]). The authors' laboratory uses the LexA-containing vector, pBTM116mod, a derivative of pBTM116 ***(19)***, and the ER DBD-containing vector, pBL1mod, a derivative of pBL1 ***(21)***, which contain *TRP1* and *HIS3* selection markers, respectively (*see* **Fig. 1**). Various bait cDNAs encoding full-length or truncated NRs (RXR, RAR, ER, progesterone receptor, vitamin D receptor, thyroid hormone receptor) have been cloned in these vectors. Several reports of successful two-hybrid library screens with these baits have been published ***(3–8)***.

2.1.2. AD Vector

This vector, which directs the synthesis of proteins fused to an AD, is designed on the same scheme as the DBD vector (*see* **Fig. 2**). The most com-

monly used ADs include the AD of the yeast GAL4 protein ***(1,17,18)***, the AD of the herpes virus protein, VP16 ***(19)***, and B42, an activating sequence from *E. coli* ***(20)***. Fusion proteins containing these ADs are generated by subcloning-appropriate cDNAs in-frame into the multiple cloning site. They are typically expressed under the control of constitutive (*ADH1*, *PGK*) or inducible (*GAL1*) promoters. This laboratory uses pASV3mod, a derivative of pASV3 ***(21)***, which contains the VP16 AD, a nuclear-localization sequence, and a *LEU2* selectable marker (*see* **Fig. 2**). Several prey sequences encoding full-length, truncated, or mutated nuclear receptors (RAR, RXR, TR, ER, VDR, PR) have been cloned in pASV3mod ***(3–8)***.

2.2.3. Library of AD Hybrids

This library consists of genomic or cDNA sequences isolated from an organism, a tissue, or a cell line, which are fused to the sequence encoding the AD in the AD vector. A variety of libraries are available both commercially or directly from laboratories where they have been constructed. The authors have constructed mouse embryo cDNA and yeast genomic DNA libraries in the vector λ ASV3 ***(21)***. These libraries have been used successfully to isolate proteins that bind to retinoid receptors and estrogen receptors ***(4–8)***.

2.2. Yeast Reporter Strains

The yeast strains used for two-hybrid screens contain one or more reporter genes whose expression is detected by growth on a selective medium (*HIS3*, *LEU2*, or *URA3*) or by a colorimetric assay (*LacZ*). The use of strains containing multiple reporters facilitates the elimination of false positives obtained in two-hybrid screens. The authors' laboratory uses the L40 and PL3 reporter strains ***(19,22)***. L40 has the following genotype: *trp1 leu2 his3 ade2 LYS2::(lexAop)$_{4x}$-HIS3 URA3::(LexAop)$_{8x}$-LacZ*. The *TRP1* and *LEU2* markers select for the yeast transformants containing pBTM116mod and pASV3mod, respectively. Interacting clones are selected by virtue of their ability to grow on histidine-deficient media containing 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3* gene product, at a concentration inhibitory to the growth of the cells expressing only the bait. Putative positive clones are then identified as those that also turn blue in the β-galactosidase assay. The genotype of the PL3 reporter strain is: *ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::(ERE)$_{3x}$-URA3*. This strain contains a *URA3* reporter gene driven by three ER-binding sites. The *HIS3* and *LEU2* markers select for transformants containing pBL1mod and pASV3mod. Interaction is detected on uracil-deficient media containing 6-azauracil (6-AU), an inhibitor of the *URA3* gene product (orotedine-5'-monophate decarboxylase [OMPdecase]), and can be assayed quantitatively by determining the OMPdecase activity. Note that it is useful to

have two different reporter systems based on different DBDs, and in which reporter genes are driven by different promoters (*see* **Note 1**).

2.3. Yeast Media

A variety of media are required for the maintenance and selection of yeast reporter strains used in the two-hybrid system: rich medium (YPD) for growing cultures and defined minimal media (synthetic dextrose [SD] and synthetic complete medium [SC]) for maintaining selection for plasmids. Most yeast strains have a doubling time of 90 min in YPD medium and approx 140 min in minimal media during the exponential phase of growth. Media are used either as liquid broth or as solid medium containing 2% agar. All media are made up in distilled water and autoclaved at 120°C and 15 Ib/in^2 (15 min for 1 L media). Yeast can be stored at –70°C in 15% (v/v) glycerol.

1. YPD (YEPD): bacto-yeast extract (1%) (10 g/L), bacto-peptone (2%) (20 g/L), glucose (2%) (20 g/L), (bacto-agar [2%] 20 g/L for plates only).
2. SD (synthetic dextrose minimal medium): bacto-yeast nitrogen base without amino acids (0.67%) (6.7 g/L), glucose (2%) (20 g/L), (bacto-agar [2%] 20 g/L for plates only).
3. SC (synthetic complete medium): bacto-yeast nitrogen base without amino acids (0.67%) (6.7 g/L), glucose (2%) (20 g/L), drop-out mix (0.2%) (2 g/L) (bacto-agar [2%] 20 g/L for plates only).
4. Drop-out mix: A combination of the following ingredients minus the appropriate supplements used to maintain selection (usually tryptophan, leucine, histidine, and uracil). It should be mixed thoroughly by turning end-over-end for at least 15 min with a clean, dry mortar and pestle. Ingredients: adenine (0.5 g), alanine (2.0 g), arginine (2.0 g), asparagine (2.0 g), aspartic acid (2.0 g), cysteine (2.0 g), glutamine (2.0 g), glutamic acid (2.0 g), glycine (2.0 g), histidine (2.0 g), inositol (2.0 g), isoleucine (2.0 g), leucine (4.0 g), lysine (2.0 g), methionine (2.0 g), *para*-aminobenzoic acid (0.2 g), phenylalanine (2.0 g), proline (2.0 g), serine (2.0 g), threonine (2.0 g), (2.0 g), tyrosine (2.0 g), uracil (2.0 g), valine (2.0 g). Store in a clean, dry bottle. Drop-out mixes are also available commercially (Bio 101/Anachem).
5. 3-AT plates: Make a stock solution of 1 *M* 3-AT (Sigma no. A-8056). Sterilize by filtration. Add 3-AT to minimal media after autoclaving and cooling to 55°C. Typically, 3-AT is used at concentrations of 1–50 m*M*, depending on the yeast reporter strain and the DBD vector used (*see* **Note 2**).
6. 6-AU plates: Make a stock solution of 2 mg/mL 6-AU (Sigma no. A-1757). Sterilize by filtration. Add 6-AU to minimal media after autoclaving. Typically, 6-AU is used at concentrations of 3–60 μg/mL.

2.4. Reagents

2.4.1. Yeast Transformation

1. 1 *M* LiAc: Dissolve 10.2 g LiAc (Sigma no. L-6883) in 90 mL dH_2O. Adjust the final volume to 100 mL and filter sterilize.

2. 50% PEG (w/v): Dissolve 50 g PEG, mol wt 3350 (Sigma no. P-3640) in 30 mL deionized water and adjust the final volume to 100 mL. Mix well by inversion. This solution is filter-sterilized using a 0.45-µm filer unit (Nalgene) and stored in an airtight bottle (*see* **Note 3**).
3. 2 mg/mL Carrier DNA: Dissolve 200 mg high-quality salmon-sperm DNA (Sigma no. D-1626) in 100 mL TE buffer (10 m*M* Tris-HCl, pH 8.0, 1.0 m*M* ethylenediamine tetraacetic acid [EDTA]). Disperse the DNA into solution by drawing it up and down in a 10-mL pipet. Leave overnight at 4°C on a magnetic stirrer to obtain a homogeneous solution. If required, shear the DNA by sonicating briefly. A small aliquot (500 ng) is analyzed by agarose-gel electropheresis to estimate the average size of the preparation, which should be approx 7 kb, but ranges from 2 to 15 kb. Oversonication (with an average size closer to 2 kb) drastically decreases the transformation efficiency. Aliquot the DNA and store at –20°C. Prior to use, denature the DNA by boiling for 10 min in a 100°C water bath. Then immediately transfer the DNA to an ice water bath.

2.4.2. Preparation of Protein Extracts for Immunoblot Analysis

1. Breaking buffer: 0.4 *M* KCl, 50 m*M* Tris-HCl, pH 7.9, 1 m*M* phenylmethylsulfonyl fluoride, Protein inhibitor cocktail, 2.5 µg/mL (leupeptin, pepstatin, aprotinin, antipain, chymostatin).
2. Acid-washed glass beads (Sigma no. G-8772; 0.45 mm).

2.4.3. β-Galactosidase Overlay Assay

1. 0.5 *M* Potassium phosphate buffer, pH 7.0: Mix 61 mL of 1 *M* K_2HPO_4 (228 g/L H_2O) and 39 mL 1 *M* KH_2PO_4 (136 g/L H_2O), and add H_2O to final volume of 200 mL. Filter-sterilize and store. Mix prior to use: 93 mL 0.5 *M* phosphate buffer, 6 mL *N*,*N*-dimethyl-formamide (DMF), and 1 mL 10% sodium dodecyl sulfate (SDS).
2. X-Gal stock solution (20 mg/mL): Dissolve 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactopyramoside (X-gal) (Boehringer no. 745 740) in 50 mL DMF and store at –20°C in the dark.
3. Low-melting agarose (Gibco-BRL Ultrapure LMP agarose no. 15517-022).

2.4.4. Quantitative β-Galactosidase Liquid Assay

1. Z buffer: 16.1 g $Na_2HPO_4 \cdot 7H_20$ (60 m*M* final), 5.5 g $NaH_2PO_4 \cdot H_2O$ (40 m*M* final), 0.75 g KCl (10 m*M* final), 0.246 g $MgSO_4 \cdot 7H_20$ (1 m*M* final), 3.5 mL β-mercaptoethanol (50 m*M* final) in 1 L H_2O, adjusted to a final pH of 7.0. Store at 4°C. Do not autoclave.
2. *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/mL): Dissolve 200 mg of ONPG (Sigma no. N-1127) in 50 mL Z buffer and store at –20°C.
3. 1 *M* Na_2CO_3 in dH_2O.
4. Chloroform ($CHCl_3$).
5. 0.1% SDS.

2.4.5. Quantitative OMPdecase Activity Assay

1. 0.5 *M* Na phosphate buffer, pH 7.0: Mix 57.7 mL 1 *M* Na_2HPO_4 and 42.3 mL 1 *M* NaH_2PO_4 and add H_2O to final volume of 200 mL.
2. 10 m*M* OMP: Dissolve 2 × 25 mg OMP (Sigma no. O-1376) in 10.2 mL sterile H_2O. Store at –20°C.
3. 10X OMPdecase buffer: Mix 40 mL 0.5 *M* Na phosphate buffer, pH 7.0 (0.4 *M* final), 0.5 mL 1 *M* $MgCl_2$ (10 m*M* final), 5 mL 10 m*M* OMP (1 m*M* final), dissolve 6.63 mg pyridoxal phosphate (0.5 m*M* final), and add H_2O to final volume of 50 mL. Make aliquots of 5 mL for 50 assays and store at –20°C.
4. [^{14}C]OMP (39.6 mCi/mmol; 0.020 mCi/mL): A NEN research product available on request.
5. Alkaline solution (Packard Instrument, Solvable NEF-910G).
6. 70% Perchloric acid (Prolabo no. 20 589.260).

2.4.6. Plasmid Rescue

1. Yeast lysis buffer: 2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1.0 m*M* EDTA.
2. Acid-washed glass beads (Sigma no. G-8772; 0.45 mm).

3. Methods

3.1. Construction of the Bait and Establishment of Two-Hybrid Screening Conditions

The first step in a yeast two-hybrid screen is to construct the bait plasmid that expresses the protein of interest or a particular domain of this protein as a fusion to the DBD. This plasmid is transformed into an appropriate yeast reporter strain, and a series of control experiments is performed to establish whether the bait is expressed as a stable fusion protein, which does not activate the reporter by itself. Based on the results, conditions for the screen are set up or different constructs and/or reporter strains are tested.

3.1.1. Construction of Bait Plasmid and Controls

If the LexA/L40- or ER/PL3-based system is used, insert the bait cDNA into the polylinker of pBTM116mod or pBL1mod, respectively. Often, the bait cDNA is amplified by polymerase chain reaction (PCR), introducing appropriate restriction sites for insertion in frame into pBTM116mod and/or pBL1mod. Using as few amplification cycles as possible is recommended in order to minimize the risk of introducing mutations. Using primers from the LexA (or ER DBD) coding sequences and *ADH1* (or *PGK*) terminator sequences flanking the polylinker sites, the inserts should be verified by sequencing (*see* **Note 4**). Introduce the bait plasmid and the empty AD vector, pASV3mod, into the appropriate yeast reporter strain (L40 or PL3). In parallel, it is advisable to

transform the reporter strain with the bait plasmid and, as positive control (if available), a pASV3mod plasmid coding for a VP16 fusion protein known to interact with the bait. This control will ensure that the assays to evaluate interaction, and to establish conditions for screen, are working properly.

3.1.2. Yeast Transformation

Plasmids can be introduced into yeast by electroporation ***(23,24)*** or by chemical treatment ***(25,26)***. The procedure given below is a variation of the high-efficiency LiAc method developed by Schiestl et al. ***(25)*** and is applicable for both yeast strains, L40 and PL3.

1. Inoculate cells from a fresh plate into 25 mL YPD and grow overnight at 30°C with shaking. Typically, inoculate at optical density $(OD)_{600}$ 0.01 in the evening to get a culture at OD_{600} 0.8–1.0 (equivalent to approx 2×10^7 cells/mL) the following morning. This culture will give sufficient cells for 10 transformations.
2. Pellet the cells by centrifugation at 3000*g* for 5 min at room temperature. Resuspend the pellet in 10 mL sterile water and centrifuge the cells again at 3000*g* for 5 min.
3. Resuspend the cell pellet in 1.0 mL sterile water and transfer the cell suspension to a 1.5 mL microcentrifuge tube. Pellet the cells at maximum speed for 15 s and remove the supernatant.
4. Resuspend the cells in 250 µL 100 m*M* LiAc and incubate the suspension at 30°C for 15 min.
5. In the meantime, boil a sample of carrier DNA (2 mg/mL) for 10 min and quickly chill on ice water.
6. For each transformation, aliquot 173 µL of the transfomation mix (freshly prepared by mixing 1.2 mL of 50% PEG [w/v], 180 µL 1.0 *M* LiAc, 250 µL carrier DNA [2 mg/mL], 100 mL water) into labeled 1.5 mL microcentrifuge tubes. Distribute 2 µL of transforming DNA (0.1–5 µg) into each tube (*see* **Note 5**). Vortex vigorously. Prepare a control tube without plasmid DNA.
7. Vortex the suspension of cells and add 25 µL of this suspension to each transformation tube. Mix by vortexing.
8. Incubate at 30°C for 30 min with shaking.
9. Heat-shock the cells in a 42°C water bath for 20 min.
10. Plate one-fortieth or one-twentieth of the cell suspension directly onto SC–minus plate, which selects for the presence of the plasmid.
11. Incubate the plates for 2–4 d at 30°C.

3.1.3. Testing Bait for Expression

Expression of the bait is analyzed by Western blot using an antibody against the N-terminal DBD moiety, the protein fused to the DBD, or an integrated epitope tag. A variety of anti-LexA polyclonal and monoclonal antibodies are available commercially (Clontech, Santa Cruz). A monoclonal antibody (F3)

against the F region epitope tag of the ER DBD can be requested from P. Chambon (Strasbourg, France). Yeast protein extracts are prepared as described below:

1. Inoculate the yeast strain containing the bait plasmid into 15 mL SC-minus medium and grow overnight at 30°C to OD_{600} = 0.8.
2. Collect the cells by centrifugation at 3000*g* for 5 min. Resuspend the cell pellet in 1.0 mL sterile H_2O and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
3. Spin for 15 s and resuspend the pellet in 150 µL breaking buffer.
4. Add approximately the same volume of glass beads until the beads reach a level just below the meniscus of the liquid. Place each sample on ice.
5. Vortex vigorously for 30 s and return to ice to cool. Repeat four times for each sample.
6. Centrifuge at 4°C for 15 min at maximum speed.
7. Transfer the supernatant to a new microcentrifuge tube, determine protein concentration, and load 50–100 µg/lane on a polyacrylamide gel electrophoresis gel (*see* **Note 6**).

3.1.4. Testing Bait for Autonomous Activation

Before performing a two-hybrid screen, the level of reporter activation by the bait protein itself should be known, and whether any background activation can be suppressed. Ideally, the L40 reporter strain that expresses the LexA fusion protein should not grow on selective medium lacking His supplemented with 3-AT, and the colonies should be white in the presence of X-Gal. The PL3 reporter strain that expresses the ER DBD fusion protein should not grow on selective medium lacking uracil supplemented with 6-AU, and the colonies should not express OMPdecase activity to a significant degree.

3.1.4.1. Growth Assay for L40

1. Grow the L40 transformant containing the bait-LexA plasmid and the empty pASV3mod vector overnight on SC-Trp-Leu plate.
2. A loopful of cells is scraped and resuspended in 1 mL sterile H_2O. Titer the cell suspension by using serial dilutions and counting cells on an hemocytometer.
3. Plate approx 10^5, 10^6, and 5×10^6 cells onto SC-Trp-Leu-His plates (140 × 140 mm) containing increasing concentrations of 3-AT (1, 3, 10, 30, and 50 m*M*). In addition, plate about 500 cells onto SC-Trp-Leu plate as a control of growth.
4. Incubate at 30°C.
5. Count the colonies on each plate after 2–5 d. Calculate the frequency of His^+ colonies, i.e., a ratio of the number of colonies on His-deficient plates over the number of colonies on plates that contain His. This will give an indication of the background that will be encountered in the screen.

3.1.4.2. β-Galactosidase Overlay Assay

1. Grow L40 transformants containing the bait-LexA plasmid and pASV3mod on SC-Trp-Leu plate for 1–3 d as either patches or single colonies. Include a negative control and a positive control (e.g., a known interactor), if available.
2. Prepare 0.5% low-melting agarose in 0.5 *M* potassium phosphate buffer, pH 7.0, 6% DMF, 0.1% SDS. For a standard 90-mm-diameter plate, add 8–10 mL of agarose, cooled to about 60°C, and add β-mercaptoethanol to 50 m*M* and X-Gal to 0.5 mg/mL. Overlay plates (pour the agarose slowly from one spot on the edge of plate until all cells are covered). The DMF, SDS, and β-mercaptoethanol will help to permeabilize the cells.
3. After the agar has solidified, the plates are inverted and incubated at 30°C. Monitor for color changes. Activation of the LacZ will give a blue color in a few hours (*see* **Note 7**).

3.1.4.3. Quantitative β-Galactosidase Assay

There are two methods for measuring β-galactosidase activity from yeast. In the first method, a cell extract is prepared, and the activity is normalized to the amount of protein assayed. In the second method, the cells are permeabilized to allow the substrate to enter the cells, and the activity is normalized to the number of cells assayed. The former method is more accurate when comparing cells under different conditions of growth.

3.1.4.3.1. Method I: Assay of Cell Extract

1. Grow 15-mL cultures to OD_{600} = 0.5 to 1.0 (~2×10^7 cells/mL).
2. Centrifuge cells at 3000*g* for 5 min at 4°C. Resuspend the cell pellet in 1.0 mL sterile water and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
3. Spin for 15 s, discard supernatant, and resuspend the pellet in 150 mL Z buffer.
4. Add approximately the same volume of glass beads until the beads reach a level just below the meniscus of the liquid. Place samples on ice. Vortex vigorously for 30 s and return to ice to cool. Repeat four times for each sample.
5. Centrifuge at 4°C for 15 min at maximum speed.
6. Transfer 5–50 μL of the protein extract (or Z buffer for the blank) to a fresh tube and adjust to 500 μL with Z buffer. Vortex and equilibrate in a water bath at 30°C for 5 min.
7. Initiate the reaction by adding 100 μL of 4 mg/mL ONPG. Mix and begin timing. Incubate at 30°C until a pale yellow color has developed.
8. Stop the reaction by adding 250 μL 1 *M* Na_2CO_3 and note time. Measure OD at 420 nm.
9. Measure the protein concentration in the extract using the dye-binding Bradford reagent (Bio-Rad Laboratories).
10. Calculate the specific activity of the extracts according to the following formula:

$$\text{Specific Activity (nmol/mg/min)} = \frac{OD_{420} \times 0.85}{0.0045 \times \text{Protein} \times \text{Volume} \times \text{Time}}$$

where Protein is the protein concentration of the yeast extract in mg/mL, Volume is the extract volume assayed in mL, and Time is the time of reaction in minutes.

3.1.4.3.2. Method II: Permabilized Cell Assay

1. Grow 5 mL cultures to mid-log phase (2×10^7 cells/mL).
2. Centrifuge and resuspend cells in 5 mL Z buffer, then place on ice.
3. Determine OD_{600} for each sample by diluting 0.5 mL cell suspension in 0.5 mL Z buffer.
4. Set up two reaction tubes for each sample: Use 1 mL cells and mix 0.1 mL cells with 0.9 mL Z buffer.
5. Add 50 µL $CHCl_3$ and 20 µL 0.1% SDS. Vortex vigorously for 15 s.
6. Preincubate the sample at 30°C for 5 min.
7. Start the reaction by adding 0.2 mL 4 mg/mL ONPG.
8. Stop the reaction by adding 0.5 mL 1 *M* Na_2CO_3 when the sample has developed a pale yellow color. Note the reaction time.
9. Remove the cell debris by centrifugation for 10 min.
10. Determine OD_{420} supernatant. Calculate units using the following formula:

$$\text{Miller Units} = \frac{OD_{420} \times 1000}{OD_{600} \times \text{Volume} \times \text{Time}}$$

where Volume is the volume of the culture assayed in mL, and Time is the reaction time in minutes.

3.1.4.4. Growth Assay for PL3

1. Grow PL3 transformants containing the bait-ER DBD plasmid and the empty pASV3mod vector overnight on SC-His-Leu plate.
2. A loopful of cells is scraped and resuspended in 1 mL sterile water. Count the cells on an hemocytometer.
3. Plate approx 10^5, 10^6, and 5×10^6 cells onto SC-His-Leu-Ura plates (140 × 140 mm) containing increasing concentrations of 6-AU (3, 10, 30, and 60 µg/mL). In addition, plate about 500 cells onto SC-His-Leu plate as a control to verify the estimated colony number.
4. Incubate at 30°C.
5. Count the colonies on each plate after 2–5 d. Calculate the frequency of Ura^+ colonies, i.e., a ratio of the number of colonies on Ura^- plates over the number of colonies on plates that contain Ura. Extrapolate from this frequency the number of colonies that would be obtained in an actual library screen and determine if this is a background level that can be tolerated (*see* **Note 8**).

3.1.4.5. Quantitative OMPdecase Assay

OMPdecase activity can be determined by measuring the release of $^{14}CO_2$ from [^{14}C]OMP as described below ***(29)***.

1. Grow 15-mL cultures to OD_{600} = 0.5–1.0 (~2×10^7 cells/mL).
2. Centrifuge cells at 3000*g* for 5 min at 4°C. Resuspend the cell pellet in 1.0 mL 0.1 *M* Na phosphate buffer, pH 7.0, and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
3. Spin for 15 s and resuspend the pellet in 150 µL breaking buffer: 0.1 *M* Na phosphate buffer, pH 7.0, 6 m*M* β-mercaptoethanol, 0.1 m*M* OMP (freshly prepared before use by mixing 0.6 mL 0.5 *M* Na phosphate buffer, pH 7.0, 1.2 µL β-mercaptoethanol, 30 µL 10 m*M* OMP, and 2.4 mL H_2O for 20 assays).
4. Add approximately the same volume of acid-washed glass beads (0.45 mm), until the beads reach a level just below the meniscus of the liquid. Place each sample on ice.
5. For each sample, label a conical glass vial containing a center well (Bibby Sterilin, no. 1190/04M), and prepare a 25-mm diameter glass microfiber filter GF/C (Whatman, no. 1822,025). Each filter is folded and inserted into a plastic cap (Sarstedt, no. 65.809.499). Distribute 100 µL alkaline solution all over the filter. Place the cap with the filter into the vial outside the center well (without touching the glass with the filter).
6. To break yeast cells, vortex each sample vigorously for 30 s and return to ice to cool. Repeat four times.
7. Centrifuge at 4°C for 15 min at maximum speed.
8. In the meantime, fill the center well of each vial with 1 mL 1X OMPdecase buffer containing [^{14}C]OMP (prior to use, mix 1.8 mL 10X OMPdecase buffer, 16 µL [^{14}C]OMP, and 16.2 mL H_2O for 20 assays).
9. 2–20 µL protein extract (or breaking buffer for the blank) are added to the reaction mixture at time zero, and the vial is capped with a rubber bung. Swirl gently to mix and incubate at room temperature for 30 min.
10. Stop the reaction by adding 0.2 mL 70% perchloric acid. Cap the vial immediately after addition. The [^{14}C]O_2 produced is released from the reaction mixture by acidification of the sample and is collected as carbonate on the alkaline solution-soaked filter.
11. After at least 3 h, the filter is transferred to a scintillation counter vial with 5 mL Ready Solv-Safe scintillation fluid. 15 µL of [^{14}C]OMP-containing 1X OMPdecase buffer is applied to an alkaline solution-soaked filter, and the filter is transferred to 5 mL scintillation fluid. Vortex each scintillation vial vigorously for 1 min three times.
12. Measure the protein concentration in the extracts using the dye-binding assay of Bradford.
13. Calculate OMPdecase specific activity by using the following formula:

$$\text{Specific Activity (nmol/mg/min)} = 1.5 \times \frac{\text{cpm}_{\text{filter}} - \text{cpm}_{\text{blank}}}{\text{cpm}_{\text{reaction mix}} - \text{cpm}_{\text{blank}}} \times \frac{1}{\text{Time}} \times \frac{1}{\text{Protein}} \times \frac{1}{\text{Volume}}$$

where $\text{cpm}_{\text{filter}}$ is the cpm observed for each sample, $\text{cpm}_{\text{reaction mix}}$ is the cpm in the 15 µL reaction mixture which contain 1.5 nmol OMP, Time is the reaction time in minutes (30 min), Protein is the protein concentration of the yeast extract in mg/mL, and Volume is the extract volume assayed in mL.

3.2. Screening for Interacting Proteins

A strategy for screening libraries in L40 is outlined below. The authors recommend screening the library by performing a two-step selection. In this protocol, the library (*see* **Note 9**) is first introduced into the bait-containing reporter strain, and the library transformants that express interacting proteins are then selected. A more rapid, but inferior, alternative approach is to perform a one-step selection by plating the library transformation mix directly on plates that select for both the presence of the library plasmid and reporter activation (*see* **Note 10**).

3.2.1. Library Transformation

For a representative mammalian cDNA library, approx 10^6–10^7 transformants need to be screened. Therefore, it is recommended to perform small-scale pilot transformations of the reporter strain with the library and to optimize transformation conditions before proceeding to a full screen. This will facilitate scaling-up the experiment to obtain the desired number of total transformants.

1. Streak a yeast colony containing the bait plasmid on SC-Trp plate and incubate overnight at 30°C (s*ee* **Note 11**).
2. Inoculate cells in 250 mL YPD at OD_{600} 0.01 and grow overnight with shaking at 30°C until the culture reaches an OD_{600} = 0.8–1.0 (equivalent to approx 2×10^7 cells/mL).
3. Centrifuge cells for 5 min at 3000*g* at room temperature. Wash the cells with 30 mL sterile water.
4. Resuspend the cells in 1.5 mL 100 m*M* LiAc and incubate the suspension at 30°C for 15 min.
5. In the meantime, boil the carrier DNA 2 mg/mL for 10 min and quickly chill on ice.
6. To a separate tube, add the components of the transformation mix in the order listed and vortex after each addition: 12 mL PEG (50% w/v), 1.8 mL 1.0 *M* LiAc, 1 mL sterile water, 2.5 mL carrier DNA (2 mg/mL), and 0.25 mL library DNA (1 mg/mL) (*see* **Note 12**).
7. Pellet the cells and discard the supernatant.
8. Add the transformation mix to the cell pellet and vortex vigorously to resuspend the cell pellet.
9. Incubate at 30°C for 30 min.
10. Heat shock at 42°C for 20 min and mix by inversion every 5 min to facilitate heat transfer.
11. Collect the cells by centrifugation and remove the supernatant with a micropipet.
12. Gently resuspend the cell pellet in 30 mL sterile H_2O, and plate onto 100 SC-Trp-Leu plates (140 × 140 mm). The total number of transformants should be calculated by plating 3 μL on SC-Trp-Leu.

13. Incubate the plates for 3–5 d at 30°C. Typically, 5×10^6–10^7 transformants are obtained by this protocol.

3.2.2. Collection of Primary Transformants and Screening for Interacting Proteins

1. Pour 2 mL sterile water on each plate and gently scrape yeast transformants off the plate with a sterile glass spreader. Repeat with another 2 mL. Pool transformants from 10 plates (a total of ~5×10^5 clones) into sterile 50 mL tubes.
2. Dilute 10 µL of each pool into 1 mL water and calculate the cell titer by using an hemocytometer.
3. Plate a number of cells corresponding to 10× the number of transformants in each pool (~5×10^6 cells) on 140-mm SC-Trp-Leu-His + 3-AT plates (*see* **Notes 13** and **14**). In parallel, plate approx 500 cells on SC-Trp-Leu to determine the number of viable cells. Centrifuge the remaining cells, and resuspend the pellet in the same volume of 65% glycerol (v/v), 10 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$. Mix well by vortexing at low speed. Freeze 1 mL aliquots at –70°C.
4. Incubate the plates at 30°C for 5 d (*see* **Note 15**).
5. At d 5, pick colonies and streak onto selective SC-Trp-Leu-His + 3-AT plates. Incubate the plates at 30°C.
6. Colonies that grow under histidine selection are next tested for LacZ expression using the BG overlay assay described in **Subheading 3.1.4.2.** Colonies that are His^+ $LacZ^+$ will be further characterized as first-round positives (*see* **Note 16**).

3.2.3. Library Plasmid Isolation

1. Centrifuge 5–10 mL of a saturated culture grown on selective medium and resuspend the cell pellet in 1 mL sterile H_2O. Transfer to 1.5 mL centrifuge tube.
2. Collect the cells by centrifugation for 15 s at full speed and resuspend the pellet in 100 µL of lysis buffer (2% Triton X-100, 1% SDS, 0.1 *M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA).
3. Add 100 µL phenol/CHCl3 and approximately the same volume of glass beads (0.45 mm) until the beads reach a level just below the meniscus of the liquid.
4. Vortex vigorously for 2 min and keep on ice for at least 15 min.
5. Spin at full speed at 4°C for 15 min.
6. Use 2 µL to transform by electroporation a $leuB^-$ *E. coli* strain (HB101).
7. Plate on minimal M9/ampicillin plates (supplemented with proline and thiamin chloride, in the case of HB101, but lacking leucine) and incubate at 37°C for 3 d. Colonies that grow on these plates contain the pASV3mod library plasmid, because the yeast *LEU2* gene carried by this plasmid can complement the bacterial $leuB^-$ mutation. A less-stringent selection method is to first plate transformations on LB/ampicillin plates, then restreak on M9 medium.
8. Prepare DNA miniprep from an isolated colony. Minipreps can then be restriction-digested with *ClaI*, and the size of inserts determined on an agarose minigel (*see* **Note 17**).

3.2.4. Elimination of False Positives

1. Retransform purified library plasmids into the yeast host strain, L40, in combination with the original bait plasmid or with control plasmids encoding nonrelated baits. Plate transformants on SC-Trp-Leu plates. Assay transformants for growth on SC-Trp-Leu-His (+ 3-AT) and for β-galactosidase activity.
2. Library-encoded proteins that activate on their own or in the presence of nonrelated baits are considered false positives and are discarded. A table of commonly identified two-hybrid false positives has been compiled and made available on the World Wide Web, at http://www.fccc.edu:80/research/labs/golemis/Table1.html.
3. If appropriate, perform additional control tests in a different two-hybrid system: If the library screen has been performed with the LexA/L40-based system, transform the isolated library plasmids into the PL3 reporter strain with the ER DBD-bait fusion or nonrelated DBD fusions (*see* **Subheading 2.2.**). Plate transformants on SC-His-Leu plates. Assay transformants for growth on SC-His-Leu-Ura (+ 6-AU) and for OMPdecase activity.

3.2.5. Sequence Analysis and Database Searches

1. Sequence the library insert of the positive isolates, determine the reading frame of the coding sequence and perform a BLAST search of the cDNA sequence against protein databases ***(30)***.
2. If the isolated cDNA is truncated and corresponds to a novel sequence, the next step is to clone the full-length cDNA by using conventional cDNA library screening or RACE approaches ***(31)***. Note that a TBLASTN search of the nucleotide sequence against expressed sequence tag-containing databases may provide additional sequences rapidly. The size of the transcript should be determined by Northern analysis.
3. Once the sequence of the complete cDNA is obtained, the databases can be searched for regions of similarity that resemble conserved motifs or functional domains of known proteins. The goal is to obtain answers to questions of what function(s) the interacting protein has, which may be of interest with respect to the biological relevance of the interaction. The authors suggest using the following protocol for searching databases ***(32)***:
   a. Split sequences into overlapping 200–300-residue segments.
   b. Use a program such as BLASTP or FASTA to search a protein database for sequences similar to those in the segments.
   c. A match having >25% identity over 80 residues is usually significant. For weaker homologies, screen the database again with the matching sequence to find related sequences and perform alignments to detect conserved residues.
   d. Conserved regions can be used to search further with PROFILESEARCH.
   e. Compare the sequences to known motifs in the PROSITE database.
   f. Use any biological information about the conserved regions to determine an analogous function for the test protein.

3.3. Subsequent Characterization of Interacting Proteins

Once the full-length cDNA corresponding to a specific interacting protein has been obtained, the next step is to show that the interaction occurs under physiological conditions and is relevant to the function of the partner. The following includes a few issues of obvious importance that should be addressed:

1. Does the full-length bait interact with the full-length interacting protein? In many cases, the library insert encodes only a segment of the putative interacting protein. Occasionally, the interaction detected may result from the exposure of a protein–protein interface that is not normally available for interaction in the context of a full-length protein. In this extreme case, full-length proteins will not interact with each other.
2. Does the bait interact directly with the library cDNA-encoded protein? The two-hybrid interaction detected could potentially involve an endogenous yeast protein that is bound to the bait. Thus, the interaction has to be examined in vitro using purified epitope (e.g., glutathione-*S*-transferase, His, Flag)-tagged proteins expressed from either *E. coli* or baculovirus-infected insect cells ***(33)***.
3. Do the bait and the novel interacting protein associate in vivo? First, it is important to determine if the interacting protein is expressed in an appropriate cell type and intracellular compartment to function in conjunction with the bait protein. Second, coimmunoprecipitation experiments should be performed from a cell in which both proteins are expressed to investigate their association in vivo.
4. Does the interaction require the same amino acids in the bait that are essential for its biological activity? For instance, NRs possess well-defined transcriptional ADs (*see* **Subheading 1.**). If a good correlation can be established by mutagenesis between the ability of the receptor to activate transcription and its ability to interact with a given protein, then the interaction may be of biological importance. In this respect, it is of interest to test the effects of specific agonistic and antagonistic ligands on the interaction.
5. Does the interacting protein affect the biological activity of the bait? Overexpression of a potential receptor-interacting protein (wild-type or dominant-negative mutants) in cultured cells interferes with the transactivation potential of the receptor which is a good indication for a biological relevant interaction. The ultimate assay to probe the functional importance of this interaction is to use gene targeting to inactivate or modify the gene encoding the novel partner in mice or cell lines, which can differentiate in response to ligand (*see* **Note 18**). The consequences of these mutations on cell differentiation and gene induction upon treatment with specific NR ligands will be investigated, and any alteration in the phenotype of the treated mutant mice or cell lines, when compared to wild-type, will indicate that the novel protein is involved in nuclear receptor function.

4. Notes

1. The authors have found it beneficial to test bait activities in both L40 and PL3 before choosing which system to use for the screen because the sensitivities of the two systems are different. In the authors' experience, the LexA/L40-based system is usually, but not always, more sensitive than the ER/PL3-based system. The less sensitive PL3 strain and the related PL1 strain, in which the URA3 reporter is driven by a single ER binding site ***(22)***, may be more suitable for screens with baits that have significant intrinsic transcriptional activity. When a large number of positive clones are obtained from a primary screen, it is beneficial to retest them in the second two-hybrid system to readily eliminate false positives.
2. The level of background *HIS3* expression and the amount of 3-AT required to suppress the residual growth on minimal media without His is dependent on the reporter strain and DBD vector used. The L40 strain transformed by pBTM116mod has no significant background after 5 d at 30°C. However, a low level expression of *HIS3* caused by the LexA fusion protein is encountered with some baits, which is sufficient to allow growth without His. The minimal level of 3-AT required to restore His auxotrophy is established by introducing the DBD-bait plasmid and an empty AD vector into the strain and plating the transformation mix directly or transformants on selective medium containing increasing concentrations of 3-AT. Background colony number is scored after 5 d at 30°C (*see* **Subheading 3.1.4.1.**).
3. Small variations above or below the PEG concentration optimum in the transformation mix, which is 33% (w/v), can reduce the number of transformants.
4. For sequencing cDNAs in pBTM116mod from the 5' end, the authors use a primer derived from the LexA sequence, 63 bp upstream of the *Eco*RI site: 5' CGTCAGCAGAGCTTCACC 3'. The primer the authors used for sequencing from the 3' end is from the *ADH1* promoter, 108 bp downstream of the *Pst*I site: 5' TTAAAACCTAAGAGTCAC 3'. The primer for sequencing cDNAs in pBL1mod from the 5' end is derived from the ER DBD sequence, 40 bp upstream of the *Xho*I site: 5' ATGTTGAAACACAAGCGC 3'. The primer for sequencing cDNAs in pASV3mod from the 5' end is derived from the VP16 sequence, 36 bp upstream of the *Xho*I site: 5' TGAGCAGATGTTTACCGATG 3'. The primer for sequencing cDNAs in pBL1mod and pASV3mod from the 3' end is from *PGK* terminator, 47 and 40 bp downstream of the *Eco*RI and *Bgl*II sites, respectively: 5' CTGGCAATTCCTTACCTTCC 3'.
5. Two plasmids, such as the DBD-bait plasmid and an AD vector, can be cotransformed into a single cell by including both plasmids in the same transformation mix. However, the efficiency of transformation is reduced. An alternative approach is to introduce the DBD-bait plasmid first, then retransform the strain with the AD vector.
6. A fast method to confirm expression of the bait protein by Western blot is the following ***(27)***:
   a. Grow yeast cells until OD_{600} = 0.8.

b. Spin cells (1.5 mL) for 5 min in a microcentrifuge.
c. Add 50 µL 2X Laemmli sample buffer (10% β-mercaptoethanol, 6% SDS, 20% glycerol, 0.025X stacking buffer, 0.2 mg/mL bromophenol blue) to the pellet, vortex, and freeze at –70°C.
d. Transfer frozen samples directly to a boiling water bath or to a PCR machine set to cycle at 100°C. Boil 5 min.
e. Centrifuge 5 s and load on gel.

7. The cells that are used for the β-galactosidase overlay assay can be easily recovered. Pick the colonies or patches through the top agar and streak them on fresh plates. Despite the permeabilization, the cells are still viable.
8. Compare the background levels obtained in the LexA/L40 and ER/PL3 reporter systems. From this comparison, decide which reporter system is the most appropriate for the library screen. In general, the authors give preference to the most sensitive reporter system, even if it may be prone to background problems; in some cases, use of the most stringent reporter system eliminates detection of biologically relevant interactions ***(28)***.
9. The authors recommended to use a library from a tissue source in which the bait protein is known to be biologically relevant.
10. Such selection minimizes the number of plates required to screen a large number of transformants. However, the authors prefer the two-step protocol, because it allows to screen the clones with a factor of multiplicity and hence increases the probability of isolating cDNAs whose expression may result in a weak activation of the reporter.
11. For best results, the bait plasmid should have been introduced into the L40 strain less than ~7 d prior to transformation with the library.
12. Adjust the volume of library DNA, but keep the combined volume of sterile water and library DNA constant in the transformation mix.
13. Not all cells expressing interacting proteins plate at 100% efficiency on His^- selective medium. Thus, to maximize chances of isolating these cells, each primary transformant should be represented on the selection plate by 3–10 individual cells. Although this protocol may result in redundant isolations of the same cDNAs, it will guarantee that all primary transformants are represented by at least one cell on the selective plate.
14. In a screen for proteins that interact with NRs in a ligand-dependent fashion, add the appropriate ligand to the plates at the desired concentration, typically 10^{-6} *M* for β-estradiol, t-RA, and 9-*cis*-RA.
15. Colonies should appear in 2–5 d. To keep the plates from drying out after 2 d, put parafilm around each plate. Observe the plates every day and mark colonies on the plate with a dot of a given color using a permanent marker. At d 5, pick all colonies and streak by day of appearance. This will facilitate the decision of which clones to analyze first.
16. $LacZ^+$ phenotype is less sensitive than His^+ phenotype. Thus, some biologically relevant weak bait–prey interactions may activate HIS3 and allow L40 to grow in the absence of His, but may not activate LacZ and cause the strain to turn blue in

the presence of X-Gal. For this reason, colonies that are His^+ $LacZ^-$ should also be further characterized (as second-round positives).

17. To identify redundant clones prior to bacterial transformation, yeast miniprep DNA can be used as template in PCR reactions with primers derived from sequences in the library plasmid flanking the cDNA insertion site (*see* **Note 4**). PCR products are then digested with a restriction enzyme that cuts frequently (i.e., *Hae*III). Analysis of the digestion products and nondigested PCR products on a 1.5% agarose gel should indicate which cDNAs are identical.
18. The F9 murine embryonal carcinoma cell line is an example of a well-established model system for the study of RA functions at the cellular and molecular level ***(34)***. These cells differentiate into three distinct endodermal cell types upon treatment with RA, and a number of genes are known to be differentially expressed during this process.

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16

Isolation of a p300/CBP Cointegrator-Associated Protein Coactivator Complex

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1. Introduction

Nuclear hormone receptors belong to a large family of structurally related proteins that include the steroid, retinoic acid, and vitamin D receptors. These receptors function as ligand-activated transcription factors and regulate complex programs of gene expression involved in growth and differentiation of many tissues. Recent studies have established that binding of hormone to nuclear hormone receptors induces a conformational change in the receptor, which facilitates the recruitment of transcriptional coactivator proteins.

The nuclear receptor coactivator/steroid receptor coactivator (NCoA/SRC) family of proteins was initially identified biochemically based on their ability to interact with ligand-bound estrogen receptor α (ERα) ***(1)***. Three distinct but related family members of NCoA/SRC proteins have been identified and cloned. Including NCoA-1) or steroid receptor coactivator 1 (SRC1), NCoA-2 or GRIP1/TIF2 ***(2–4)***, and the p300/CBP cointegrator-associated protein (p/CIP), also known as ACTR/AIB1/RAC3/TRAM-1/SRC-3 ***(5–10)***.

In addition to interacting with liganded nuclear hormone receptors, NCoA/SRC proteins are capable of interacting with other proteins that function as coactivators such as CBP, CARM-1, and p/CAF. This suggests that NCoA/SRCs serve a scaffold-like function for the assembly of multiprotein complexes. However, the majority of studies to date have utilized techniques involving overexpression and coimmunoprecipitation of recombinant proteins; consequently, little is known regarding the identity of endogenous factors that interact with NCoA/SRC proteins. This chapter outlines a protocol (**Fig. 1**) for

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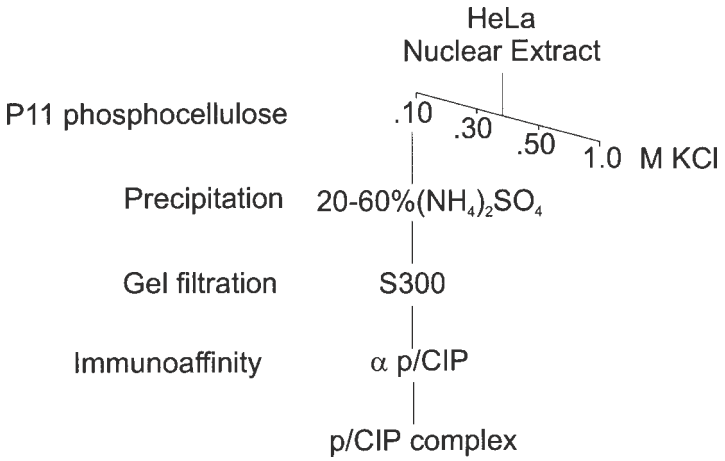

Fig. 1. Purification protocol. Schematic representation of the various chromatographic steps used to purify the p/CIP complex.

purifying a large macromolecular complex containing p/CIP and associated proteins. In addition, the chapter describes a glutathione-*S*-transferase (GST)-pulldown assay and a histone acetyltransferase activity assay that can be used to monitor the biochemical and functional activity of the coactivator complex through the various purification steps.

2. Materials

2.1. Nuclear Extract Preparation

1. HeLa cells grown to mid-log phase.
2. Hypotonic lysis buffer: 20 m*M* HEPES, pH 7.9, at 4°C, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* DTT, 0.2 m*M* PMSF, 10 μg/mL each of leupeptin, aprotinin, and 2 μg/mL pepstatin.
3. Nuclei resuspension buffer: 20 m*M* HEPES, pH 7.9, at 4°C, 0.24 *M* sucrose, 20 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* DTT, 0.2 m*M* PMSF, 10 μg/mL each of leupeptin, aprotinin, and 2 μg/mL pepstatin.
4. Nuclei isolation buffer: Same as nuclei resuspension buffer except that the KCl is increased to 1.2 *M*.
5. Dialysis buffer: 20 m*M* Tris-HCl, pH 7.9, at 4°C, 5% glycerol, 100 m*M* KCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 1 m*M* DTT.
6. PBS, trypan blue 0.4% (Gibco).
7. Refrigerated low speed centrifuge and Dounce homogenizer.

2.2. Column, Resins, and Buffer Used in Purification Scheme

2.2.1. P11 Phosphocellulose Chromatography

1. 0.5 *M* HCl; 0.5 *M* NaOH; and 20 m*M* Tris-HCl, pH 7.9.

2. Equilibration buffer A: 20 m*M* Tris-HCl, pH 7.9, 100 m*M* KCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 10% glycerol, 0.5 m*M* DTT, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.
3. Funnel and filter paper (Whatman).

2.2.2. Gel Filtration Chromatography

1. Sephacryl™ S-300 (Pharmacia) high resolution column.
2. Fast protein liquid chromatography.
3. Equilibration buffer B: 20 m*M* Tris-HCl, pH 7.9, 100 m*M* KCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 10% glycerol, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.
4. Ultrafree-4 centrifugal filter unit (Millipore).
5. Refrigerated tabletop centrifuge.

2.2.3. Affinity Column Preparation

1. Crosslinking buffer: 0.2 *M* $NaHCO_3$, pH 8.3, 0.5 *M* NaCl.
2. Dialysis Cassette Slide-A-Lyzer 3.5K (Pierce).
3. Wash buffer A: 0.5 *M* ethanolamine, pH 8.3, 0.5 *M* NaCl.
4. Wash buffer B: 0.1 *M* Na-acetate, pH 4.0, 0.5 *M* NaCl.
5. Neutralizing buffer: 20 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl.
6. HiTrap *N*-hydroxysuccinamide (NHS) Sepharose column (Pharmacia).
7. Protein A Sepharose CL 4B (Amersham).
8. Solid dimethylpalmilidate (DMP) (Sigma).
9. 0.2 *M* Na borate, pH 9.0.
10. 0.2 *M* Ethanolamine, pH 8.0.
11. PBS, Na azide 0.1%.
12. Peristaltic pump.

2.2.4. Immunoaffinity Chromatography

1. Peristaltic pump.
2. Equilibration buffer: 20 m*M* Tris-HCl, pH 7.9, 100 m*M* KCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.
3. Wash buffer: 20 m*M* Tris-HCl, pH 7.9, 0.3 *M* KCl, 0.2 m*M* PMSF, 0.1% Triton X-100, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.
4. Elution buffer: 0.1 *M* glycine, pH 3.0, 0.1 *M* NaCl.
5. 1 *M* Tris-HCl, pH 8.0

2.3. Ammonium Sulfate Precipitation

1. Solid Bio-ultra pure grades of ammonium sulfate.
2. Refrigerated centrifuge.
3. Ice bath, mortar and pestle.

2.4. GST-Pulldown Assay

1. Glutathione Sepharose beads.

2. NET buffer: 50 m*M* Tris-HCl, pH 7.6, 0.3 *M* NaCl, 5 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.
3. NET-N buffer: 50 m*M* Tris-HCl, pH 7.6, 0.3 *M* NaCl, 5 m*M* EDTA, 1 m*M* DTT, 0.1% NP-40, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A.

2.5. Liquid Histone Acetyltransferase Assay

1. Protein A-Sepharose resins.
2. [^{3}H]-acetylCoA (1.85 mBq, 7.7Ci/mmol, Amersham).
3. P-81 (Whatman) Phosphocellulose paper disks (2.5-cm diameter).
4. IPH buffer: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA, 0.5% NP-40, 0.1 m*M* PMSF and 5 μg/mL each of aprotinin, leupeptin, and pepstatin.
5. 25 μg Total histones and bovine serum albumin.
6. 0.05 *M* $NaHCO_3/Na_2CO_3$ buffer, pH 9.2.
7. Acetone:methanol:chloroform (1:1:1 v/v).
8. Liquid scintillation counter.

3. Methods

3.1. Preparation of Nuclear Proteins

For small-scale purification, HeLa cells are grown on 150-mm dishes to 80% confluency. Alternatively, for large-scale preparations, cells grown to mid-log phase can be obtained from the National Cell Culture Center (Minneapolis, MN). Nuclear extracts were prepared (*see* **Note 1**) using a standard method with some modifications ***(11)***.

1. Turn on the low-speed centrifuge and allow it to cool.
2. Centrifuge 20 L of Hela cells at 2000*g* for 10 min, drain off media and resuspend in prechilled PBS.
3. Wash cells with prechilled PBS in 50-mL conical tubes, centrifuge at 2000*g* for 10 min, drain the tubes thoroughly and measure the packed cell volume.
4. Resuspend in hypotonic lysis buffer and centrifuge at 2000*g* for 10 min. Cells will be swollen at this step.
5. Resuspend cells to 3× the original packed cell volume in hypotonic lysis buffer.
6. Leave on ice for 10 min, then homogenize slowly with 10 strokes of a dounce homogenizer using a type B pestle. Check for lysis by trypan blue exclusion (*see* **Note 2**).
7. Collect nuclei by centrifuging at 3300*g* for 10 min, remove supernatant, and resuspend in nuclei resuspension buffer to 5× original packed cell volume. Centrifuge at 10,000*g* for 30 min to obtain a tight nuclear pellet.
8. Resuspend pellet (nuclei contained) in resuspension buffer to 2× packed nuclear volume. Place the resuspended nuclei in a beaker containing a small magnetic stirrer and place the entire mixture in an ice bath.

9. While stirring slowly (over 15–20 min), add nuclear isolation buffer dropwise, until the final concentration of KCl reaches 0.42 *M* (*see* **Note 3**). Stir slowly for additional 30 min on ice.
10. Centrifuge in a JA20 rotor at 10,000*g* for 30 min at 4°C. Retain the supernatant containing the nuclear proteins.
11. Measure the volume and dialyze against equilibration buffer A.

3.2. P11 Column Preparation and Loading of the Sample

1. Weigh out the appropriate amount of P11 cellulose phosphate (*see* **Note 4**) and stir into 25 vol 0.5 *M* NaOH for 5 min.
2. Decant off the supernatant, and wash the P11 resin in a funnel with 20 m*M* Tris-HCl, pH 7.9, until the pH is below 11.0.
3. Decant and add 25 vol 0.5 *M* HCl for 5 min.
4. Decant off the supernatant and wash P11 in a funnel with 20 m*M* Tris-HCl, pH 7.9 until the filtrate pH is above 3.0
5. All subsequent steps should be performed at 4°C. Pour the stirred slurry into the column and wash with at least 2 column volume starting buffer A. The pH of the eluant should be approx 7.9.
6. Load dialyzed nuclear extract (*see* **Subheading 3.1.**) onto the P11 column at a flow rate of 0.5 mL/min and collect the flow through fraction.
7. Wash with 2 column volume buffer A containing 0.1 *M* KCl, 0.3 *M* KCl, 0.5 *M* KCl, and 1.0 *M* KCl. Collect each fraction and analyze for immunoreactive p/CIP by Western blotting.
8. Re-equilibrate the P11 column with 2 column volume starting buffer. The P11 column is reusable and stable at 4°C for 1–2 mo.

3.3. Precipitation with Ammonium Sulfate

Ammonium sulfate precipitation is used to reduce the large sample volume eluted from the P11 column. The majority of p/CIP is found in the 0.1 *M* KCl fraction. This fraction is precipitated with 0–20, 20–60, and 60–80% ammonium sulfate. We have found that approx 95% of the NCoA proteins are precipitated with 20–60% ammonium sulfate saturation.

1. Determine the protein concentration and volume of the starting material (0.1 *M* KCl fraction) and pour the mixture into a prechilled oversized glass beaker.
2. Place the beaker on ice on top of a stirrer and stir slowly (*see* **Note 5**).
3. Determine the amount of solid ammonium sulfate that will be required to obtain a 0–20, 20–60, and 60–80% saturation using a standard precipitation table. Grind the solid ammonium sulfate, using a mortar and pestle. Add the ammonium sulfate to the protein solution in small batches over a period of time to allow it to dissolve prior to adding additional ammonium sulfate (*see* **Note 6**). After addition is complete, continue stirring for an additional 30 min to allow equilibrium to be reached between the dissolved and aggregated proteins.

4. Centrifuge at 10,000*g* for 30 min at 4°C.
5. Decant and save the supernatant, record new volume for the next step and calculate the amount of ammonium sulfate will be needed as mentioned above.
6. Dissolve the precipitate in 5 mL buffer A (*see* **Note 7**).
7. Dialyze the sample against buffer A to remove residual ammonium sulfate.

3.4. Gel Filtration Chromatography Using Sephacryl S300 Column

1. Equilibrate Sephacryl S-300 column (300 mL) with 2 column volume (600 mL) equilibration buffer B at a flow rate of 0.4 mL/min (*see* **Note 8**).
2. Load the protein sample onto the column. Sample volume should be kept below 5 mL to ensure better separation on the Sephacryl S300 column (*see* **Note 9**).
3. Elute sample with 1.5 column volume buffer B (500 mL) and collect 5 mL fractions. Identify the p/CIP-containing fractions by UV absorbance at 280 nm and by immunoblotting using an anti-p/CIP antibody.
4. Pool the p/CIP containing fractions and reduce the volume of the sample to approx 20 mL using an Ultrafree-4 filter unit (Millipore).
5. Normally, p/CIP elutes from the Sephacryl S300 column in two major peaks. A 1.5 MDa, which also contains the transcriptional coactivator, CBP, and a smaller 600–700 kDa peak, which contains p/CIP as well as other N-CoA/SRC proteins.

3.5. Immunoaffinity Chromatography

Immunoaffinity chromatography is the critical step in purifying the p/CIP complex and is highly dependent on the antibodies used. Both monoclonal or affinity purified polyclonal antibodies can be used for this purpose. Ideally, the antibodies should recognize unique regions of p/CIP and should be tested for their ability to recognize p/CIP by both Western blotting and by immunoprecipitation from nuclear extracts.

3.6. Partial Purification of Anti-p/CIP from Serum

1. The p/CIP polyclonal antiserum was raised against a His-tagged recombinant p/CIP (AA 591–803).
2. The immunoglobulin G (IgG) rich fraction was purified using protein A Sepharose chromatography exactly as described ***(12)***.

3.7. Crosslinking p/CIP Antigen to Hi-Trap NHS Sepharose

1. The regions corresponding to p/CIP is amplified by PCR and subcloned into PQE 30 vector (Qiagen). The recombinant protein is expressed in bacteria and purified using Ni-NTA agarose exactly as described in the manufacturer's protocol (Qiagen).
2. Dialyze recombinant p/CIP against crosslinking buffer using a Slide-A-Lyzer 3.5K (Pierce) dialysis cassette at 4°C.
3. Wash a Hi-Trap NHS-activated column (1 mL, Pharmacia) with ice-cold 1 *M* HCl (3 × 2 mL each).

4. Wash the column once with 2 mL crosslinking buffer.
5. Measure the protein concentration of the sample and retain a small aliquot (from **step 2**). Pass at least 2 mg purified recombinant protein through the activated resin 8–10× using two syringes attached to both ends of the column with Luer adapters.
6. Incubate the column containing the bound protein at room temperature for 2 h.
7. Collect breakthrough and compare the protein concentration with original sample for coupling efficiency.
8. Wash the column with 3 mL of crosslinking buffer.
9. Wash the column sequentially with wash buffer A (3 × 2 mL each), wash buffer B (3 × 2 mL each), and with wash buffer A (3 × 2 mL each).
10. Leave the column in wash buffer A at room temperature for 15 min.
11. Now wash the column with wash buffer B (3 × 2 mL each), then with wash buffer A (3 × 2 mL each), and again with wash buffer B (3 × 2 mL each).
12. Wash the column with 2 mL neutralizing buffer.
13. The column containing p/CIP antigen is now ready for use and can be used to purify the specific p/CIP antibodies.
14. The HiTrap column containing the crosslinked protein can be stored at 4°C and is stable for at least 1 yr.

3.8. Affinity Purification of p/CIP Antibodies

1. Wash the antigen-affinity column (from **Subheading 3.6.**) with 2 mL of low pH elution buffer (0.1 *M* glycine, pH 3.0, 0.1 *M* NaCl) to wash out any uncoupled protein.
2. Equilibrate column with 10 mL of starting buffer (0.1 M Tris-HCl, pH 8.0, 0.1 *M* NaCl).
3. Pass the IgG purified anti-p/CIP antibodies (from **Subheading 3.5.**) through the antigen column by recirculating with a peristaltic pump at a flow rate of 1 mL/min for 1 h at room temperature.
4. Wash with 10 mL starting buffer.
5. Elute the affinity bound p/CIP antibodies with 5 mL low-pH elution buffer (1 mL × 5).
6. Identify the immunoglobulin-containing fractions by absorbance at 280 nm. Alternatively, the IgG can be identified by analyzing a small aliquot by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
7. Neutralize the pH of the eluants immediately by adding one-tenth volume of each collected fraction (100 µL) of 1 *M* Tris-HCl, pH 8.0
8. To regenerate the column, wash with 10 mL starting buffer.

3.9. Preparation of p/CIP Immunoaffinity Column

1. Wash 1 mL protein A Sepharose CL 4B three to four times with ice-cold PBS.
2. Incubate affinity purified anti-p/CIP antibodies (at least 2 mg antibody/mL of beads) with protein A Sepharose for 1 h at room temperature.
3. Wash beads twice with 10 vol 0.2 *M* sodium borate, pH 9.0.

4. Resuspend beads in 10 vol 0.2 *M* sodium borate, pH 9.0. Save 10 μL from this step to assess the binding efficiency.
5. Add 0.05 g solid DMP to the 10-mL slurry.
6. Incubate for 1 h at room temperature with gentle rocking.
7. Wash the beads once with 0.2 *M* ethanolamine, pH 8.0, and incubate the beads in 0.2 *M* ethanolamine, pH 8.0, for 2 h at room temperature with gentle mixing.
8. Wash beads with PBS containing 0.1% sodium azide (2 × 10 mL). Remove 10 μL equivalent of beads to check the coupling efficiency.
9. Check the efficiency of coupling by boiling the aliquots of beads taken before and after crosslinking with DMP, run the samples on an SDS-PAGE gel (10%) followed by Coomassie blue staining.

3.10. Affinity Purification of p/CIP Complex

1. Equilibrate a control affinity column containing an irrelevant antibody (e.g., rabbit IgG) crosslinked to protein A Sepharose, and the anti-p/CIP affinity column with 10 vol of starting buffer (20 m*M* Tris, pH 7.9, 100 m*M* KCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 0.2 m*M* PMSF, 5 μg/mL each of leupeptin, aprotinin, and pepstatin A).
2. Pass the Sephacryl-purified p/CIP containing fractions (from **Subheading 3.4.**) through the control antibody column at a flow rate of 0.2–0.4 mL/min. Alternatively, the sample can be precleared by adding 1 mL control affinity resin and incubating for 1 h at 4°C. Retain the supernatant.
3. Pass the precleared supernatant (from **step 2**) through the anti-p/CIP affinity column at a flow rate of 0.2–0.4 mL/min, and recirculate the eluate five times. Wash the beads extensively with 20–30 vol wash buffer.
4. Elute the retained proteins with elution buffer and collect 400-μL fractions into tubes containing 40 mL 1 *M* Tris-HCl, pH 8.0, to bring pH back to physiological levels. Collect 10 fractions.
5. Analyze a 10-μL aliquot of each fraction by Western blotting. Normally, p/CIP elutes in the first three fractions.
6. Purification efficiency of the p/CIP complex at each step can be monitored by Western blotting using an equal amount of protein sample (5–10 μg) derived from nuclear extract (*see* **Subheading 3.1.**), P11-100 m*M* KCl fraction (*see* **Subheading 3.2.**), Sephacryl S-300 fractions (*see* **Subheading 3.4.**) and anti-p/CIP affinity column elution (*see* **Subheading 3.10.**). Each purification step ensures an enrichment of p/CIP containing complex (**Fig. 2**).
7. Alternatively, the affinity-purified p/CIP complex can be separated by SDS-PAGE gel and proteins associated with this complex are visualized by silver staining (**Fig. 3**).
8. After elution, regenerate the column immediately by washing with 10 mL starting buffer.
9. Column is stable for up to 1 yr at 4°C.

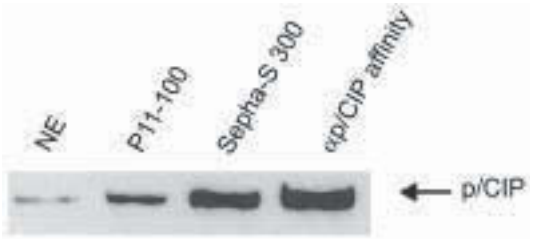

Fig. 2. Enrichment of p/CIP at the various purification steps. Western blot analysis of elutes derived from the nuclear extract (NE), 100 m*M* P11 fraction, Sephacryl S300, and anti-p/CIP affinity column. For each sample, 10 μg protein were separated by SDS-PAGE, transferred to nitrocellulose and then probed with specific antibodies to p/CIP.

3.11. Histone Acetyltransferase Assay

Functional properties of the coactivator complex through all purification steps are retained as the ability of p/CIP complex is measured by their intrinsic histone acetyltransferase activity performed as below:

1. Resuspend 20 μg purified proteins in 500 μL IPH buffer.
2. Add 50 μL anti-p/CIP affinity resin and incubate for 1 h at 4°C.
3. Pellet immuncomplexes by gentle centrifugation and wash three times with 1 mL IPH buffer.
4. After the final wash, the complex is resuspended in 50 μL IPH buffer containing either 25 μg free histones or bovine serum albumin.
5. The reaction is initiated by adding 1 μL of [^{3}H]-acetylCoA and incubating at 30°C for 30 min.
6. The entire reaction is spotted onto a P-81 phosphocellulose paper disk.
7. Soak paper disks extensively with 50 mL 0.05 *M* $NaHCO_3/Na_2CO_3$ buffer, pH 9.2, and allow to stand in buffer for 30 min at 37°C.
8. Wash the disk three times with acetone:methanol:chloroform (1:1:1 v/v) and dry on blotting paper.
9. Measure [^{3}H]-acetyl incorporation using a liquid scintillation counter.

3.12. GST-Pulldown Assay

To assess whether the affinity purified p/CIP complex retains the ability to interact with liganded nuclear hormone receptors a GST pulldown assay can be employed using bacterially expressed nuclear hormone receptors.

1. Generate recombinant GST fusion proteins containing the ligand-binding domain of GST-estrogen receptor (AA 251–595) or GST-retinoic acid receptor (RAR) (AA 143–462) according to standard protocols ***(5)***.

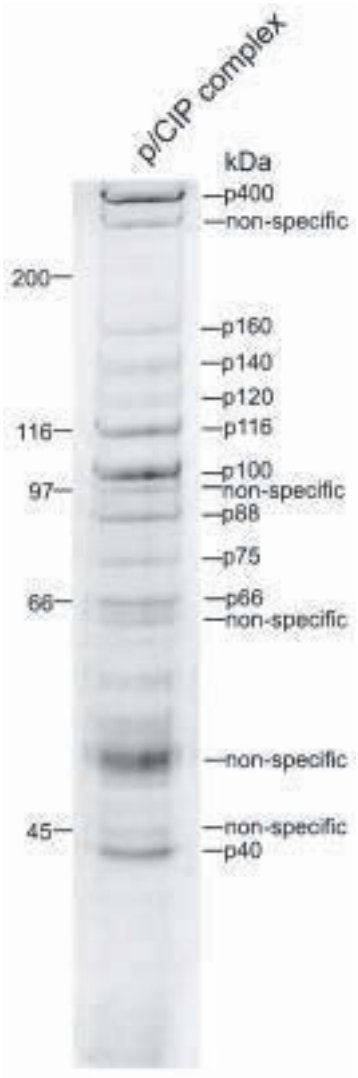

Fig. 3. The p/CIP complex contains multiple proteins. Silver staining of SDS-PAGE gel containing an aliquot of p/CIP complex eluted from an anti-p/CIP affinity column. Approximate molecular weight of the isolated peptides are indicated on the right. Molecular weight standards are indicated on the left of the complex.

2. Bind 10 μg GST-recombinant protein to 50 μL glutathione Sepharose beads twice with 500 μL NET buffer.
3. After the final wash, the buffer was aspirated down to 90 μL.
4. Add 10 μL of 10^{-6} *M* β-estradiol (Sigma) or retinoic acid (Sigma) to GST-ER or GST-RAR, respectively (final concentration 10^{-7} *M*).

5. Maintain a control experiment in the absence of ligand.
6. Incubate for 30 min at room temperature.
7. Add 20 μg purified p/CIP complex in a total volume of 200 μL NET buffer.
8. Incubate 1 h at 4°C with gentle rocking and wash the beads three times in NET-N buffer.
9. Dissolve beads in SDS-PAGE sample buffer, boil at 95°C for 3 min, and analyze the proteins bound with liganded receptors by Western blotting with the appropriate antibodies such as anti-p/CIP.

4. Notes

1. During the biochemical purification, protein samples should be kept at 4°C. To prevent proteolytic degradation the number of freeze–thaw steps should be minimized. Moreover, freshly made protease inhibitors should be used and PMSF should be added immediately before use.
2. Proper lysis of the cells ensures good extraction of proteins. The percentage of cells lysed after homogenization should be greater than 90% when stained with trypan blue.
3. When calculating the total amount of KCl to add, the authors use a stock solution of resuspension buffer containing 1.2 *M* KCl, so that the dilution factor to obtain a final KCl concentration of 0.42 *M* is 2.8. *P* = the total volume of nuclei resuspension buffer; *X* = amount of 1.2 *M* KCl buffer to be added (mL), then:

$$(P + X) \div X = 2.8$$

4. About 1 mL (equivalent to 0.25 g dry resin) of wet slurry of P11 phosphocellulose resin is required to purify 10–12 mg proteins. Wet slurry can be stored at 4°C with 0.5 *M* phosphate buffer, pH 7.0.
5. Because ammonium sulfate precipitation requires time, add freshly made PMSF to a final concentration of 0.1 m*M* and 5 μg/mL trypsin inhibitor. Stir gently to avoid foaming and denaturing of the protein.
6. Adding the ammonium sulfate in small batches, allowing each to dissolve before adding the next to prevent unwanted proteins from precipitating. Check the pH occasionally with litmus paper to make sure no drastic pH changes have occurred.
7. Dissolve protein pellet in a buffer compatible with the next purification step. Undissolved particulate, resulting from denatured protein, can be removed by centrifugation.
8. DTT may decrease the binding efficiency of crosslinked beads to the protein complex as well as shortening the life-span of the affinity column. Therefore, DTT should not be added to buffers prior to the affinity purification step (i.e., Sephacryl S-300).
9. Large volumes of dilute proteins can pose a problem using gel filtration chromatography. However, protein concentration should not exceed 50 mg/mL. Samples should be free from particulate matter before loading on to the column. For good separation, the volume ratio of the column matrix and sample to be fractionated should be between 50–100.

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Nonradioactive Photoaffinity Labeling of Steroid Receptors Using Western Blot Detection System

Simon J. Evans and Frank L. Moore

1. Introduction

Photoaffinity labeling is the process of covalently crosslinking a photoactive ligand, which can be detected postcoupling to a receptor or binding protein. This technique has proven useful in the identification of novel proteins or to further study and characterize known proteins. Photoaffinity labeling of novel receptors and binding proteins can provide information about biochemical properties of the target protein by allowing it to be traced through various separation schemes. For example, visualizing a labeled protein following one- or two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can provide information about its apparent molecular weight (mol wt) and isoelectric point ***(1,2)***. Knowledge of these biochemical characteristics, and others, can be useful in further design of purification strategies. Photoaffinity labeling of known receptors or binding proteins can be used to study important residues in the target protein's binding site. For example, photoaffinity labeling, followed by an enzymatic digest and sequencing of the labeled peptide(s), allows the identification of specific labeled residues in or near the binding pocket ***(3)***. Other studies might also use photoaffinity labels to induce a chronic activation or inactivation of a receptor by crosslinking the ligand to the binding site.

Photoaffinity labeling of receptors is accomplished by UV crosslinking a photoactive ligand to the target protein of interest. Typically, radiolabeled photoactive ligands have been used and the labeled protein(s) visualized by autoradiography following resolution by SDS-PAGE (for a review of steroid photochemistry, *see* **ref. *4***). However, this chapter discusses a novel strategy

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using a cold (nonradioactive) photoactive steroid ligand. Regardless of strategy, important issues to consider when designing a photoaffinity labeling experiment include ligand specificity, ligand detectability, ligand crosslinking efficiency, and ligand availability or ease of synthesis. The first three of these issues need to be addressed with empirical experimentation, which is discussed below. The fourth issue, availability of a good photoaffinity ligand, can be problematic. For the steroid receptor biologist, several radioactive photoaffinity steroidal ligands are commercially available (Amersham Pharmacia Biotech, Piscataway, NJ; New England Nuclear, Boston, MA) and may serve the purpose of the researcher. In other cases, however, a good ligand is not available and must be synthesized. In-house synthesis of radioligands can be undesirable or impractical, and contracted synthesis can be very expensive.

The method outlined in this chapter can provide an alternative strategy to easily synthesize a good nonradioactive photoaffinity ligand for a moderate cost. This method was developed for the photoaffinity labeling of a membrane glucocorticoid receptor (mGR) ***(2)*** found in the brains of an amphibian, the roughskinned newt (*Taricha granulosa*), but the basic protocol can be useful for the study of many different steroid receptors. This technique uses steroids with a carboxymethyloxime (CMO) modification. These molecules can be easily coupled to azido-amines with simple chemistry, purified, and used directly as photoaffinity labels. Labeled proteins can be resolved by SDS-PAGE, transferred to a membrane support and visualized by a Western blot methodology, with an antibody directed against the steroid ligand. This strategy is attractive for three reasons: Many different CMO–steroids are commercially available, and it is therefore likely that an appropriate ligand can be found; antibodies against most steroids are also commercially available, because of their popular uses in radioimmunoassays; current detection strategies employing chemiluminescence (CL) are sensitive and allow the detection of femptomol levels of labeled protein.

2. Materials

2.1. Equipment

Nonstandard laboratory equipment necessary to perform the experiments described in this chapter include a lyophilizer, Speed-Vac concentrator, a UV light source (handheld or transilluminator), thin layer chromatography apparatus, and a protein gel electrophoresis system.

2.2. Reagents and Supplies

Special reagents and supplies include: C_{18} thin layer chromatography plates and C_{18} syringe cartridges (Fisher Scientific, St. Louis, MO), CMO-steroid

conjugates (Steraloids, Newport, RI), *N*-(2-aminoethyl)-4-azido-2-nitroaniline (Molecular Probes, Eugene, OR), specific antisteroid antibodies (Sciquest.com, Research Triangle Park, NC), chemiluminescent detection system (Amersham Pharmacia Biotech or Pierce, Rockford, IL), radiography film (Eastman Kodak, Rochester, NY).

3. Methods

3.1. Synthesis of Photoactive Steroid

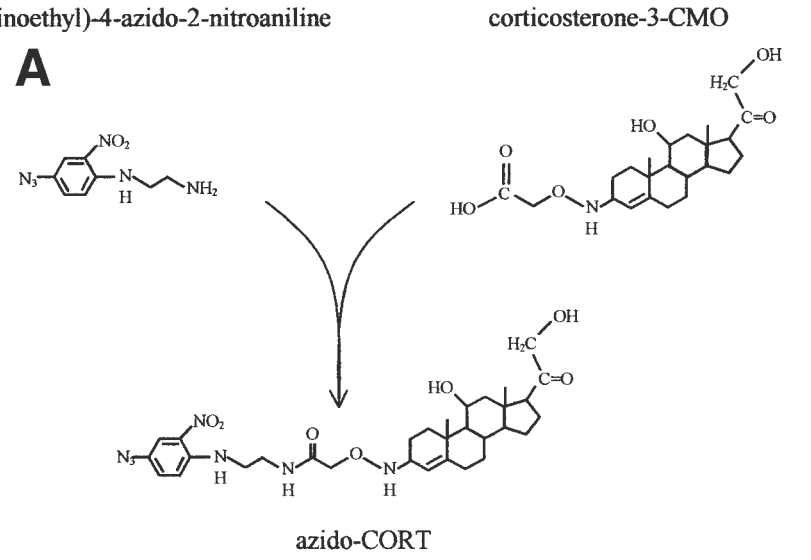
Synthesis of a photoactive steroid can be achieved by a condensation reaction between the carboxyl group of a CMO-derivitized steroid and the amine group of an azido-amine. The reaction is catalyzed by a carbodiimide in an amine-free solvent. Care should be taken during all steps involving an azide to minimize exposure to light, because these molecules are light-sensitive.

The first step toward synthesis of a photoactive steroid ligand is to select an appropriate precursor molecule. A large selection of steroids derivitized with a CMO linkage is available from Steraloids. These derivatives usually contain the CMO linkage at the 3-position of the A ring or on the side chain. Since these linkages are at opposite faces of the steroid molecule, the probability increases that one of them will not interfere with the activity of the steroid ligand at the receptor's binding site. The CMO-steroids that are the most closely related congeners of those steroids known to have the desired activity should obviously be tested first in the assay method being employed. For example, in our case 3-CMO-corticosterone provided good activity in displacing [^{3}H]corticosterone from the binding site of the membrane GR (**Fig. 1B**) and was thus utilized for the synthesis of a photoactive corticosterone (azido-CORT) (**Fig. 1A**).

The second important component of a photoaffinity label synthesis strategy is the azido-amine. A good azido-amine should have an electronegative group, which will act as an electron sink, near the azide group. This will enhance reactivity and crosslinking efficiency of the photolabel. A good azido-amine molecule for use with CMO-steroids is *N*-(2-aminoethyl)-4-azido-2-nitroaniline (**Fig. 1A**), available from Molecular Probes.

Once the reactants are identified, they can be used in a simple condensation reaction to achieve synthesis of the photolabel (**Fig. 1A**). A sample protocol used for the synthesis of azido-CORT is given below, but solvents and reactant concentrations may need to be optimized for the synthesis of other azido-steroids (*see* **Note 1**).

1. Dissolve *N*-(2-aminoethyl)-4-azido-2-nitroaniline in 30% dioxane to a concentration of 20 m*M*.
2. Dissolve CMO-steroid in 30% dioxane to a concentration of 10 m*M*.



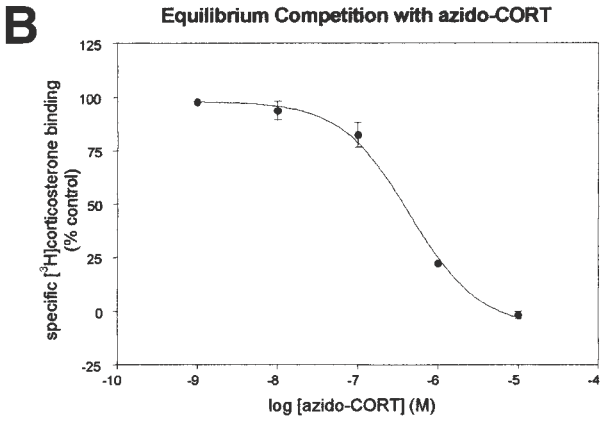


Fig. 1. Structure and activity of azido-CORT. (**A**) Diagram of *N*-(2-aminoethyl)-4-azido-2-nitroaniline and corticosterone-3-CMO, which were used as reactants in the synthesis of the azido-CORT photolabel. (**B**) Activity of azido-CORT in competition radioligand binding assays with mGR. Binding conditions were as follows: 25 m*M* HEPES, pH 7.45, 10 m*M* $MgCl_2$, 0.5 n*M* [^{3}H]corticosterone, 100 μg neuronal membrane protein in total assay volume of 0.3 mL. Azido-CORT competitor was present at concentrations given on the graph. 10 μ*M* Corticosterone was used in control tubes to define nonspecific binding. Assays were incubated at approx 25°C for 4 h in the dark then terminated by rapid filtration over GF/C filters.

3. Mix together reactants in equal volumes, then add one-tenth vol 1 *M* aqueous *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC).
4. Let the reaction proceed overnight at 4°C.

At this point, the reaction should be complete and the photolabel product can be purified away from the reactants and resuspended in a suitable solvent for use.

3.2. Purification of Photolabel Product

Purification methods need to be empirically determined. A reverse-phase purification method should work well for most steroids in the reaction described above (*see* **Note 2**). The azido-amine and the water-soluble carbodiimide are significantly more hydrophilic than most steroids and the azido-steroid product should be of intermediate hydrophilicity.

Once appropriate conditions have been defined for the separation of reactants from product a syringe-driven C_{18} cartridge can be used to purify the photolabel. For this, the reaction must be dried down and resuspended in the purification solvent, loaded onto an equilibrated C_{18} cartridge and purified. Collect small fractions (e.g., 0.5 mL for an initial 1 mL load), and run aliquots on C_{18} TLC plates to determine which fractions contain purified product. If the product fractions are still not completely free of reactants, the purification can be repeated.

It may be necessary to precede the cartridge purification with an additional extraction step. For example, ethyl acetate was used to remove a significant quantity of the reactants after azido-CORT synthesis prior to cartridge purification, making the final purification step more efficient. Again, the efficacy of such extractions must be empirically determined and can be evaluated by TLC. An example of the protocol used for purification of azido-CORT is given below.

1. Extract reaction three times with equal volumes of ethylacetate and discard organic phase.
2. Concentrate aqueous phase to dryness using a lyophilizer or Speed-Vac.
3. Resuspend reaction in 1 mL 60% acetonitrile and purify product over a C_{18} cartridge with a 60% acetonitrile isocratic gradient, collecting 0.5-mL fractions.
4. Concentrate fractions to dryness in a Speed-Vac and evaluate by C_{18} TLC.
5. Pool fractions containing pure product into a preweighed tube and concentrate to dryness again.
6. Weigh the tube with the dried product for an estimate of product mass.
7. Resuspend product in an appropriate solvent for use in assays (e.g., 100% ethanol) or resuspend in 60% acetonitrile for a second purification if necessary. Store final product at –20°C in a dark tube.
8. Product identity should be confirmed by mass spectrometry if possible.

3.3. Evaluation of Photolabel Efficacy

For discussions in this subheading, it is assumed that the photolabel being produced will be used as a photoaffinity ligand for a steroid receptor. In this case, the photoaffinity label should first be evaluated for its efficacy as a ligand. For this, the photolabel can be used as a competitor in a radioligand binding assay to gain an estimate of affinity or as a ligand in some functional assay to gain an estimate of EC_{50}. In either case, the reactions should be performed in the dark to prevent crosslinking of the photolabel to the receptor binding site, which would compromise the interpretations of the results from these experiments.

After determining the affinity (or EC_{50}) of the azido-steroid ligand, studies should evaluate the efficiency of the crosslinking between the photolabel and the binding site. This can be accomplished with the following steps: Incubate the photoactive ligand with the receptor or binding protein, expose the ligand–receptor complex to UV light, wash out the unreacted photolabel, then determine the percentage of remaining active binding sites (relative to appropriate controls) using a radioligand binding assay or functional assay. Each of these steps should be evaluated and optimized as discussed below.

First, the kinetics of UV crosslinking must be determined. The photolabel should be incubated with the receptor at sufficient concentration to achieve occupation of >90% of the receptor-binding sites (based on affinity estimation from above studies). After allowing the system to come to equilibrium (assuming no UV-independent crosslinking), the reactions can be subjected to varying lengths of UV light treatment (*see* **Note 3**). The appropriate time-course for this experiment will depend on the intensity of the light source being used. For handheld UV light sources, an initial evaluation of 1–20 min is probably sufficient. For more intense light sources, shorter time-points should be evaluated.

After UV exposure, the unreacted photolabel must be washed out (*see* **Note 4**) and radioligand binding assays or functional assays performed to determine what percentage of the receptor-binding sites have been crosslinked and unavailable for binding. This is accomplished by comparison to parallel control samples that went through a mock photolabeling and washing process. **Figure 2** shows the crosslinking efficiency of azido-CORT using a strategy similar to that described above.

3.4. Detection of Photolabeled Receptor by Western Blot

The final step of the photolabeling experiment is detection. For the methodology being described in this chapter, detection is achieved by a Western blot technique using a primary antibody directed against the steroid being used. A detailed description of Western blotting methodology is not described in this

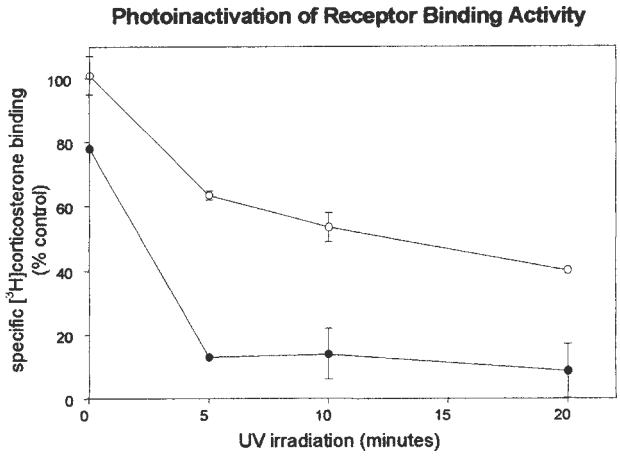


Fig. 2. Photoinactivation of mGR by azido-CORT. Neuronal membrane suspensions containing mGR were incubated with or without azido-CORT for 4 h at 25°C, then exposed to UV light for times indicated on the graph. Membrane suspensions were pelleted by centrifugation at 20,000*g* and resuspended in 25 m*M* HEPES, pH 7.45. Washing was repeated a total of three times. Radioligand binding assays were then performed (as described in **Fig. 1** legend) using [^{3}H]corticosterone to label sites and 10 μ*M* cold corticosterone to define nonspecific binding. Results were normalized to the control time tissue (no azido-CORT) at the zero UV irradiation time-point. Reprinted from *Journal of Steroid Biochemistry and Molecular Biology* **75,** Evans, S. J., Murray, T. F., and Moore, F. L. Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain. Copyright 2000, with permission from Elsevier Science ***(2)***.

chapter because this is a standard technique, and can be found in other sources ***(5–7)***. Briefly, the procedure includes resolution of proteins, after photolabeling, by SDS-PAGE; transfer of resolved proteins to a membrane support; probing the membrane-bound proteins with a primary antibody (against the epitope of interest); probing membranes, labeled with primary antibody labeled proteins, with peroxidase-conjugated secondary antibody; incubating membranes with a fluorescent peroxidase substrate (*see* **Note 5**); visualizing labeled proteins by exposure to film.

For most of the major natural steroids, commercial antibodies are available, because of their use in radioimmunoassays (Sciquest.com has a searchable antibody database for antisteroidal antibodies from various manufacturers). The first step in detection is then to identify a suitable antibody. For most of the steroid–CMO conjugates, the appropriate BSA–CMO–steroid conjugate (or other protein–steroid conjugate) is also available. These are ideal tools for

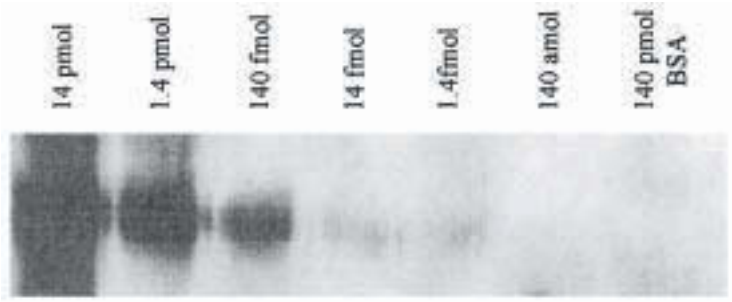


Fig. 3. Estimate of detection sensitivity of anti-CORT Western blot. BSA–CORT was blotted directly onto nitrocellulose membrane using a slot-blot apparatus. Membranes were blocked overnight with 5% gelatin in (TBS-T) (0.1 *M* Tris-HCl, pH 7.2, 0.2 *M* NaCl, 0.1% Tween), washed 3 × 10 min with TBS-T, then probed with anti-CORT neat serum (Cortex Biochemical) at a 1:1000 dilution in TBS-T. Membranes were washed 3 × 10 min with TBS-T, then probed with goat antirabbit MAb (Sigma) at a 1:20,000 dilution in TBS-T. Membranes were washed 5 × 10 min in TBS-T, then developed with SuperSignal Substrate (Pierce) and visualized by autoflourography. Amounts blotted onto membrane are given in mols CORT, except BSA control is given in mols BSA. Reprinted with permission from *Journal of Steroid Biochemistry and Molecular Biology* **75,** Evans, S. J., Murray, T. F., and Moore, F. L. Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain. Copyright 2000, with permission from Elsevier Science ***(2)***.

screening of antibodies and for use in optimization of the Western blot procedure. The BSA–CMO–steroid conjugate can be blotted onto a membrane support (e.g., PVDF or nitrocellulose) that can be used to screen a few antibodies by a Western blot detection method (*see* **Note 6**). An example of a blot containing BSA–CMO–CORT that was probed with anti-CORT antisera (Cortex Biochemical, San Leandro, CA) is shown in **Fig. 3**.

After identifying a suitable antibody the final step is to optimize the Western blot protocol. Again, a BSA–CMO–steroid conjugate (or other protein–steroid) can be used for optimization of the Western blot procedure. Parameters that need to be optimized are to identify a suitable blocking buffer (e.g., 5% BSA, 5% gelatin, 5% nonfat milk protein), identify suitable wash conditions, and identify suitable antibody concentrations. Again, it is beyond the scope of this chapter to review optimization of Western blot procedures, which can be found elsewhere.

Once the Western blot procedure has been optimized, detection of the photolabeled receptor is possible. For this, the photolabel needs to be crosslinked to the receptor using conditions defined above. In order to discern specifically labeled proteins from those nonspecifically labeled, a control must

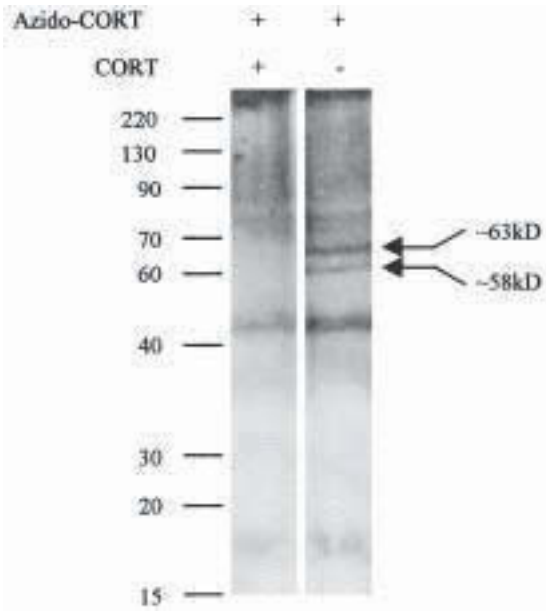


Fig. 4. Detection of azido-CORT labeled proteins. Newt neuronal membrane proteins were solubilized as previously described ***(2)*** and incubated with 1 μ*M* azido-CORT for 4 h at 25°C in 25 m*M* HEPES, pH 7.45, 10 m*M* $MgCl_2$, either in the presence or absence of 10 μ*M* cold CORT. Proteins were precipitated with ammonium sulfate, as previously described ***(2)***, resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes and processed as described in **Fig. 3** legend. Arrows indicate specifically labeled bands at 58 and 63 kDa. Reprinted from *Journal of Steroid Biochemistry and Molecular Biology* **75,** Evans, S. J., Murray, T. F., and Moore, F. L. Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain. Copyright 2000, with permission from Elsevier Science ***(2)***.

be included at the photolabeling step. This involves incubating a parallel receptor sample with photolabel in the presence of excess cold ligand to efficiently compete for the receptor's binding site so that no receptor is specifically labeled in the control. Following photolabeling, the samples must be concentrated to a small volume for loading onto the protein gel. Concentration can be achieved by precipitation and centrifugation or by ultrafiltration. Care need not be taken to retain receptor activity after the photolabeling step so harsh precipitation methods may be used (*see* **Note 7**). Finally, chemiluminescent detection of the labeled proteins may be achieved. An example of a Western blot of mGR after photolabeling with azido-CORT is shown in **Fig 4**.

At this point, the first indications of nonspecific labeling will become evident. If nonspecific labeling is unacceptable, then certain strategies need to be

employed to attempt to minimize this problem. Such strategies could include lowering the concentration of photolabel in the labeling reaction, varying the time and temperature of the labeling reaction (*see* **Note 8**) or preceding the labeling reaction with a preliminary receptor purification step if possible.

4. Notes

1. Steroids can be difficult to dissolve and usually require an organic solvent. Dioxane (up to 30%), ethanol (up to 50%), and methanol (up to 50%) have all been used successfully in the reaction chemistry. A small amount of the steroid–CMO, the azido-amine, and the carbodiimide should be tested for solubility at appropriate concentrations prior to committing larger quantities to the reaction. The solvent chosen to dissolve the reactants must have no free amines, which would compete for reaction with the carboxyl. It is also important to minimize exposure of the azide to light, both pre- and post-photolabel synthesis. Finally, the reaction should be driven by excess azido-amine to minimize the presence of unreacted CMO–steroid in the product, which could interfere with subsequent use of the photolabel.
2. TLC can be used as a fast and easy method to evaluate different purification approaches. Initially, C_{18} plates can be used to evaluate the migration of all of the reaction components and the product in a defined purification condition. For this, resuspend (dry-down, if necessary) each component (azide, EDC, steroid, complete reaction) independently in 50% acetonitrile and run on C_{18} TLC plates, using 50% acetonitrile as the mobile phase (alternatively, methanol can be used, instead of acetonitrile). The photolabel product should be easily identifiable as the predominant species in the reaction mix that is missing from the individual reactant lanes. After initial visualization, the concentration of acetonitrile can be adjusted to increase or decrease the rate of migration of the various components.
3. An efficient way to perform this experiment is to have several parallel reactions in a multiwell plate (e.g., 6-, 24-, 96-well plate), which is covered with a thick piece of paperboard. A handheld UV light source can be inverted directly onto the plate and the individual wells exposed by sliding the paperboard out, to serially expose the wells for different lengths of time, starting with the longest timepoint. This step should be performed in the cold. Typically, just placing the multiwell plate on ice with the entire apparatus on a slow shaker is sufficient.
4. If the receptor is in a suspension that can be pelleted by centrifugation, then the easiest way to wash out the label is by a few rounds of centrifugation and resuspension of the pellet in a suitable wash buffer. If the receptor is soluble, precipitation of the receptor protein, followed by centrifugation and resuspension in an appropriate buffer can be used, assuming this does not destroy the receptor's binding activity. If this is undesirable, then dialysis or ultrafiltration are usually acceptable methods.
5. Several chemiluminescent detection systems are available. SuperSignal Substrate from Pierce or Enhanced Chemiluminescence from Amersham are both very

sensitive detection systems. With most chemiluminescent detection systems, the manufacturer supplies a protocol that does not require optimization or modification.

6. The researcher must ensure that the antibodies being used do not recognize protein epitopes within the protein–steroid conjugate. BSA is often used as a carrier for antibody production and thus polyclonal antibodies might recognize BSA epitopes. If possible, the protein used as a carrier for the production of the antibody should be different from the protein being used in a protein–CMO–steroid conjugate as a substrate for the Western blot. In any case, unconjugated protein should also be blotted as a negative control to ensure no protein epitopes are being recognized, which could lead to misinterpretation of the results.
7. Many standard protein precipitation methods are possible. Acetone, at a final concentration of 80%, will efficiently precipitate most proteins. If this is used, it should be ice-cold before added to the protein solution. Alternatively, the sample can be incubated at –80°C after addition of acetone to achieve precipitation. One advantage to this method is that it will efficiently remove most detergents, which are often used when working with membrane receptors. Another standard method is precipitation with trichloroacetic acid at a final concentration of 5%. This is advantageous if volume is a concern, but care must be taken to ensure that the sample is near a neutral pH prior to running SDS-PAGE. Because most protein loading buffers have a pH indicator dye, this is easily monitored.
8. Reducing the concentration of photolabel in the photolabeling reaction could have drastic effects on background. Nonspecific labeling should decrease linearly; specific labeling should decrease based on fractional occupancy. These calculations should be possible based on estimates of affinity from **Subheading 3.3.** Also, some studies show a significant decrease in nonspecific labeling, if the tissue is frozen before and during the crosslinking process ***(8)***. In this study, the tissue is frozen at –80°C after equilibration with the photolabel, but before UV irradiation. The UV exposure then occurs while the tissue is kept frozen at –80°C by placing the tissue plate on an aluminum block that is submerged in liquid nitrogen. Following UV irradiation, the frozen tissue is allowed to warm slowly at –20°C in the dark prior to processing the tissue for electrophoresis.

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18

Analysis of Steroid Hormone-Induced Histone Acetylation by Chromatin Immunoprecipitation Assay

James R. Lambert and Steven K. Nordeen

1. Introduction

Steroid hormone receptors are members of the nuclear receptor family of ligand-activated transcriptional regulatory proteins. Recent work in the field of nuclear receptor action has demonstrated an association of receptors with coregulatory proteins termed "coactivators" and "corepressors." In the absence of hormone, or in the presence of hormonal antagonists, nuclear receptors can repress transcription through their association with corepressors. Upon binding hormonal agonists, receptors bind target sites in DNA and recruit transcriptional coactivators. In turn, coactivators, functionally, and perhaps physically, bridge DNA-bound receptors and the general transcription machinery, forming a complex capable of promoting the activation of gene expression ***(1–3)***.

A developing picture of the molecular mechanism of nuclear coregulatory protein function suggests that their roles in receptor-mediated gene expression are dependent on enzymatic activities that are either intrinsic or associated. For example, the steroid receptor coactivator 1 has been shown to possess intrinsic histone acetyltransferase activity ***(4)***. Conversely, the corepressors, NR corepressor and silencing mediator of retinoic acid and thyroid hormone receptor, have been shown to be part of a large multisubunit complex containing histone deacetylase activity ***(5–7)***. The ability of coregulatory proteins to modulate the levels of histone acetylation at target promoters thus appears to be a key step in the activation and/or repression of nuclear receptor target genes.

Increased histone acetylation has been associated with transcriptionally active genes ***(8)***. The core histones, H2A, H2B, H3, and H4, contain in their

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N-termini highly conserved lysine residues that are targets for the addition of acetyl groups by histone acetyltransferase-containing enzymes. It has been suggested that the acetylation of positively charged lysines lessens the association of DNA and histones. This loosening of the DNA wrapped around the core histones is thought to facilitate the association of transcription factors with their DNA recognition elements, leading to activation of transcription. Thus, the analysis of the state of histone acetylation at steroid hormone-responsive promoters can provide insights into the molecular mechanisms by which steroid hormone receptors act as transcriptional regulatory proteins.

This chapter describes a method that permits the analysis of histone acetylation at steroid-regulated promoters. The technique relies on the use of formaldehyde to create crosslinks between proteins and DNA in vivo. Formaldehyde is a cell-permeable compound that interacts with amino groups of protein and nucleic acids to form protein–DNA, protein–RNA, and protein–protein crosslinks ***(9–14)***. The advantage of using formaldehyde for these studies lies in the ability to efficiently reverse the formaldehyde-induced protein–DNA crosslinks, which facilitates the analysis of the DNA that was crosslinked to the particular protein of interest, in this case, histones. Cells are first treated with formaldehyde to induce protein–DNA crosslinks. The crosslinked chromatin is then isolated and used in immunoprecipitation reactions with specific antibodies to acetylated histones. The crosslinks are reversed, and the DNA is purified and subjected to quantitative polymerase chain reaction (PCR) using gene-specific primers. The degree of enrichment for the particular promoter region of interest, therefore, provides the investigator with an indication of the degree of histone acetylation in that region at a given time. A schematic of the chromatin immunoprecipitation (ChIP) assay is presented in **Fig. 1**.

2. Materials

2.1. Hormone Treatment, In Vivo Crosslinking, Harvest, and Lysis of Cells

1. Tissue culture equipment, supplies, and media for growth of cells.
2. Steroid hormone(s).
3. 37% Formaldehyde (reagent grade).
4. Phosphate buffered saline (PBS): 140 m*M* NaCl, 2.5 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH 7.5.
5. Protease inhibitors: 10 mg/mL phenylmethylsulfonylfluoride, 10 mg/mL aprotinin, 10 mg/mL pepstatin.
6. Lysis buffer: 1% sodium dodecyl sulfate (SDS), 10 m*M* ethylenediaminetetraacetic acid (EDTA), 50 m*M* Tris-HCl, pH 8.0.

2.2. Immunoprecipitation and DNA Purification

1. Immunoprecipitation dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 m*M* EDTA, 16.7 m*M* Tris-HCl, pH 8.0, 167 m*M* NaCl.

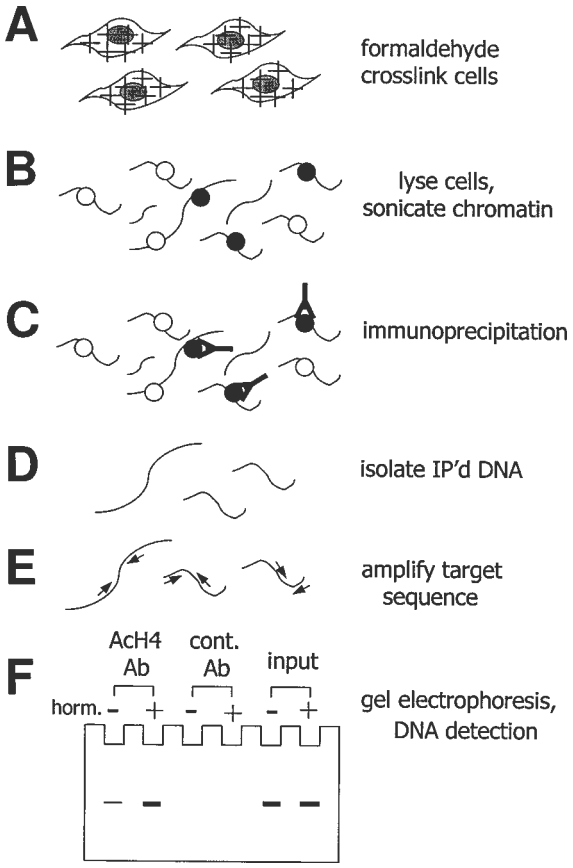

Fig. 1. Schematic diagram of the ChIP assay. **(A)** Cells are crosslinked with formaldehyde. **(B)** Cells are resuspended in lysis buffer and sonicated to produce chromatin fragments with histones covalently crosslinked to DNA (open circles represent nucleosomes with unacetylated histone H4 and black circles represent nucleosomes with acetylated histone H4). **(C)** Chromatin is immunoprecipitated with antibodies that recognize acetylated histones and the immune complexes purified on protein A agarose. **(D)** Crosslinks are reversed by heating the samples and the DNA purified. **(E)** Purified DNA is used as template in quantitative PCR reactions using primers to the gene of interest. **(F)** PCR products are resolved by PAGE and visualized by ethidium bromide staining. Increased production of the desired DNA product indicates increased histone acetylation in nucleosomes occupying that region of the genome.

2. Anti-acetylated histone antibodies (Upstate Biotechnology, cat. no. 06-599 [anti-acetylated histone H3] and 06-866 [anti-acetylated histone H4]).
3. Protein A agarose: 50% gel slurry in TE containing 0.05% sodium azide, 200 μg sonicated salmon sperm DNA, and 500 μg bovine serum albumin, for a final volume of 1 mL.

4. Low-salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 m*M* EDTA, 20 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl.
5. High salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 m*M* EDTA, 20 m*M* Tris-HCl, pH 8.0, 500 m*M* NaCl.
6. LiCl wash buffer: 250 m*M* LiCl, 1% NP-40, 1% Na deoxycholate, 1 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0.
7. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
8. Elution buffer: 1% SDS, 0.1 *M* $NaHCO_3$.
9. 5 *M* NaCl.
10. 0.5 *M* EDTA, pH 8.0.
11. 1 *M* Tris-HCl, pH 6.5.
12. 10 mg/mL Proteinase K.
13. Phenol/chloroform/isoamyl alcohol (25:24:1, v/v).
14. 3 *M* Sodium acetate, pH 5.2.
15. 20 mg/mL Glycogen.
16. Absolute ethanol.
17. 70% Ethanol.

2.3. Analysis of Immunoprecipitated DNA

1. Oligonucleotide primers: 20–26-mers with similar melting temperatures (*see* **Note 1**).
2. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl (pH 9.0 at 25°C), 1.0% Triton X-100, 15 m*M* $MgCl_2$.
3. 2 m*M* dNTP mixture (dATP, dGTP, dCTP, dTTP, each at 2 m*M*).
4. *Taq* polymerase 5 U/μL.
5. Chloroform.
6. Equipment and reagents for polyacrylamide gel electrophoresis (PAGE): Gel casting and running apparatus, electrophoresis power supply, 40% acrylamide–bisacrylamide solution (19:1), 10X TBE running buffer (0.89 *M* Tris-HCl, 0.4 *M* boric acid, 20 m*M* EDTA), TEMED, 10% ammonium persulfate (w/v), 10X sample loading buffer (30% Ficoll, 0.1 *M* EDTA, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanole FF), 10 mg/mL ethidium bromide.

3. Methods

3.1. Hormone Treatment, In Vivo Crosslinking, Harvest, and Lysis of Cells

1. Treat 2×10^6 cells on a 10-cm tissue culture dish with 10 n*M* hormone (*see* **Note 2**).
2. Crosslink histones to DNA by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubate at room temperature for 15 min on a rocking platform (*see* **Note 3**).
3. Remove media and wash cells twice with ice-cold PBS containing protease inhibitors (*see* **Note 4**). Add 5 mL ice-cold PBS containing protease inhibitors

and scrape cells with a rubber policeman. Transfer cells to a 15-mL conical centrifuge tube. Add an additional 5 mL PBS, containing protease inhibitors to the dish as a rinse and combine with the initial 5 mL of cells.
4. Pellet cells for 5 min at 1000*g* at 4°C.
5. Remove all traces of PBS and resuspend cells in 400 µL lysis buffer containing protease inhibitors. Transfer cells to a 1.5 mL centrifuge tube and incubate 10 min on ice.
6. Sonicate the lysate to reduce the DNA length to between 200 and 1000 bp (*see* **Note 5**). Place samples on ice for 20 s between pulses. Clear the lysate by centrifugation at 14,000*g* for 10 min at 4°C.

3.2. Immunoprecipitation and DNA Purification

1. Dilute the supernatant by adding to 2.5 mL immunoprecipitation dilution buffer containing protease inhibitors (*see* **Note 6**).
2. Preclear the chromatin solution by adding 80 µL protein A agarose. Rotate at 4°C for 30 min.
3. Pellet the beads by centrifugation at 500*g* for 2 min and carefully remove supernatant to a fresh tube. This represents the chromatin solution used in the immunoprecipitations.
4. Add 5 µL anti-acetylated histone H4 (or H3) antibody to 1 mL chromatin solution in a 1.5-mL siliconized centrifuge tube and incubate overnight at 4°C with rotation (*see* **Note 7**). Save the remaining chromatin solution to check the amount of input DNA after reversal of crosslinks (*see* **steps 13**, **14**, and **Note 8**).
5. Capture immune complexes by adding 60 µL protein A agarose and rotating for 1 h at 4°C.
6. Pellet beads by a brief centrifugation, remove the supernatant, and wash the beads for 5 min with 1 mL low-salt wash buffer (*see* **Note 9**).
7. Repeat **step 6**, washing with 1 mL high-salt wash buffer.
8. Repeat **step 6**, washing with 1 mL LiCl wash buffer.
9. Repeat **step 6**, washing with 1 mL TE.
10. Repeat **step 9**.
11. Remove all traces of TE by aspiration with a 28-guage needle affixed to a syringe.
12. Elute immune complexes by adding 250 µL elution buffer to beads (*see* **Note 10**). Incubate at room temperature for 15 min with rotation. Pellet beads by a brief centrifugation, then carefully transfer supernatant to a fresh tube. Repeat elution by adding an additional 250 µL elution buffer to beads and rotating 15 min at room temperature. Pellet beads and combine eluates.
13. Add 20 µL 5 *M* NaCl to the pooled eluates and incubate at 65°C for 4 h to reverse protein–DNA crosslinks.
14. Spin samples briefly in a microcentrifuge to collect any condensate and add 10 µL 0.5 *M* EDTA, 20 µL 1 *M* Tris-HCl, pH 6.5, and 5 µL 10 mg/mL proteinase K. Mix well and incubate at 45°C for 1 h.
15. Spin samples briefly in a microcentrifuge to collect any condensate and extract samples with 0.5 mL phenol/chloroform/isoamyl alcohol. Carefully remove 450 µL

aqueous layer to a fresh tube, which contains 50 µL 3 *M* sodium acetate plus 1 µL 20 mg/mL glycogen. Add 1 mL absolute ethanol, mix by inversion, and incubate at –20°C overnight.

16. Collect DNA by centrifugation at 14,000*g* for 15 min. Wash pellet with 70% ethanol and resuspend in 50 µL sterile H_2O.

3.3. Analysis of Immunoprecipitated DNA

1. Set up PCR reactions in a final volume of 50 µL by combining 50 pmol of each primer, 5 µL 10X PCR buffer, 5 µL 2 m*M* dNTP mixture, and 2.5 U *Taq* polymerase with 5 µL immunoprecipitated and input DNA (*see* **Note 11**). Control reactions consisting of no DNA and DNA containing the gene of interest serve as negative and positive controls, respectively. An additional control is to perform the PCR reactions on samples that have been generated from immunoprecipitations using a control antibody (*see* **Note 7**).
2. Overlay the samples with mineral oil if using a thermal cycler without a heated lid, place the reactions in the thermal cycler and perform PCR reactions using the following parameters: 2 min initial denaturation at 95°C, followed by 26 cycles with 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and a final extension for 5 min at 72°C (*see* **Note 12**).
3. After reactions are complete, add 150 µL chloroform to tubes, mix briefly, and centrifuge for 1 min at room temperature.
4. Remove 40 µL of the upper aqueous layer to a fresh tube, which contains 5 µL 10X sample loading buffer, and mix well.
5. Run 20 µL of the samples on a 6% polyacrylamide gel containing 1X TBE in 1X TBE gel running buffer at 150 V constant voltage (*see* **Note 13**).
6. Disassemble and stain the gel in a 0.1 µg/mL ethidium bromide solution for 10 min at room temperature on a shaking platform. Destain the gel in sterile H_2O for 10 min.
7. Document the results of the PAGE. The results of a typical analysis are shown in **Fig. 2** (*see* **Note 14**).
8. Normalize the amount of signal from the immunoprecipitated samples to the corresponding amount of input DNA by generating a ratio of immunoprecipitated sample signal:input sample signal.

4. Notes

1. The oligonucleotide primers the authors used in analysis of histone acetylation at the MMTV-luciferase promoter in T47D(C&L) cells ***(15)*** were: 5'-GCGGTTCCCAGGGCTTAAGT-3' and 5'-CCATTTTACCAACAGTACCG-3'.
2. The time of hormone treatment must be determined empirically for each particular application. A pilot experiment to see when hormone-induced gene expression can be detected should be carried out to determine the appropriate time of induction. The authors and others ***(16)*** have found that a 2-h treatment results in measurable histone H4 acetylation as determined by the ChIPs assay. For our experiments, the authors have analyzed the state of histone acetylation at the

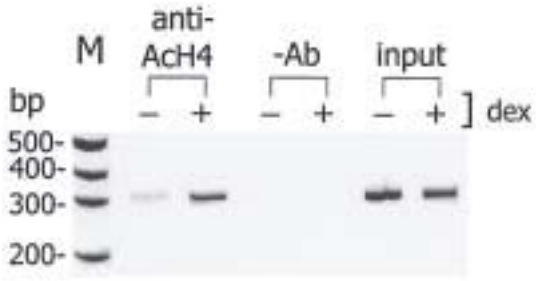

Fig. 2. Hormone-induced acetylation of histone H4. The ChIP procedure was applied to the glucocorticoid-inducible, stably integrated MMTV-luciferase gene in T47D(C&L) cells. Cells were treated with the synthetic glucocorticoid, dexamethasone (DEX, 10 n*M*), for 2 h before formaldehyde crosslinking. The ChIP procedure was applied as described using a commercially available antibody (*see* **Subheading 2.2.**) to acetylated histone H4 (AcH4 Ab). In this experiment, a mock immunoprecipitation was performed without antibody. Immunoprecipitations using irrelevant antibodies also yield no product. M, markers; bp, base pairs.

mouse mammary tumor virus (MMTV)-luciferase promoter in T47D(C&L) cells using the synthetic glucocorticoid, dexamethasone (10 n*M*) (**Fig. 2**).

3. These conditions have been optimized for the use of anti-acetylated histone antibodies available from Upstate Biotechnology and are similar to several published ChIPs protocols. The use of alternative antibodies may require varying the duration and/or temperature of formaldehyde crosslinking. Extensive crosslinking times (>30 min) should be avoided because this tends to result in the formation of cell aggregates that cannot be efficiently sonicated.
4. Where the use of protease inhibitors is indicated, the concentrations of these compounds should be 1 μg/mL.
5. Inappropriate sonication is often the cause of failure or inconsistent results. It is essential that the sonication of the chromatin produces DNA fragments of 200–1000 bp in length. Fragments that are too large are not efficiently immunoprecipitated; fragments that are too short are not efficiently amplified during the PCR reactions. Perform a pilot experiment (**Subheading 3.1., steps 1–6**) to determine the optimal conditions required to sheer the DNA into fragment sizes 200–1000 bp in length. Vary the intensity, duration, and time of sonication pulses. Take extra care to avoid foaming of the samples. If foaming occurs, decrease the power output setting and/or place the probe further into the sample. Keep the depth of the probe in the chromatin solution as constant as possible (approx 0.5 cm below the surface). Determine the lengths of DNA fragments after reversal of crosslinks (**Subheading 3.2., steps 13** and **14**) by running samples on a 1% agarose gel. The authors' experience shows that the chromatin is sheared to the appropriate length with 6–8 sets of 10-s pulses using a Branson model 450 Sonifier at power setting 2 and a duty cycle of 90.

6. The authors have found that this dilution of the chromatin solution provides for reasonable signals in the PCR analysis and allows for two samples for immunoprecipitations and one sample for both the analysis of input DNA and the degree of DNA fragmentation.
7. The use of a nonspecific antibody is recommended for initial experiments to confirm that there is little-to-no signal generated from this control immunoprecipitation. The authors have used rabbit anti-mouse immunoglobulin G and anti-hemagglutinin antibodies with the conditions described and observe no signal in the PCR.
8. The amount of input DNA in the reactions is a crucial control for the interpretation of the results. To generate the input DNA sample, perform **Subheading 3.2., steps 13–16** on 500 μL chromatin solution and resuspend the final DNA pellet in 200 μL sterile H_2O.
9. Aspiration of wash solutions must be done in such a manner to avoid loss of the protein A agarose–immune complexes. The authors find that a 200-μL pipet tip (yellow tip), affixed to a water aspirator, provides a sufficient degree of precision in the removal of the wash solutions. Simply aspirate as much of the wash solution as possible without disturbing the beads. The use of a 28-guage needle during **Subheading 3.2., step 11** will remove all of the final TE wash solution without loss of the beads.
10. Prepare elution buffer fresh.
11. The most important aspect of the interpretation of the results of the PCR is that the output of each reaction is proportional to the amount of input DNA, i.e., the reactions are quantitative. The authors have empirically determined that these volumes of input and immunoprecipitated DNA yield quantitative PCR results under the conditions described. If the signals are too faint, it may be necessary to increase the amount of DNA. If the output DNA signal is too great, input DNA can be reduced or the number of PCR cycles decreased.
12. These reaction parameters are a good starting point in the PCR. However, depending on the particular primers used, and/or the amount of DNA present, it may be necessary to alter the annealing temperature and/or number of cycles to obtain signal.
13. The electrophoresis run time will be determined by the size of the amplified DNA.
14. The authors routinely capture gel images and quantitate band intensities using the Bio-Rad Gel Doc 1000 Gel Documentation System.

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Analyzing the Contributions of Chromatin Structure in Nuclear Hormone Receptor Activated Transcription In Vivo

Christy J. Fryer and Trevor K. Archer

1. Introduction

The mouse mammary tumor virus (MMTV) promoter has been used extensively as a model system to examine the role of chromatin structure on transcriptional regulation from a steroid responsive gene (**Fig. 1**). Early studies demonstrated that the chromatin structure of the MMTV promoter was altered upon glucocorticoid treatment, such that a discrete region became hypersensitive to DNaseI ***(1)***. This hypersensitive region corresponded to the portion of the MMTV promoter that was shown to function as a hormonal enhancer in vivo ***(1)***. Examination of the MMTV promoter stably maintained in mouse mammary cell lines revealed that the MMTV long terminal repeat (LTR) organized into a phased array of six precisely positioned nucleosomes **(A–F)** ***(2–4)***. The proximal promoter, containing the hormone response elements (HREs), and binding sites for nuclear factor 1 (NF1), TATA binding protein (TBP), and octamer transcription factors (OTFs) is encompassed by nucleosomes A and B ***(2,5)***. Analysis of glucocorticoid activation of the MMTV promoter demonstrated that the glucocorticoid receptor (GR) initiated a cascade of events that led to chromatin disruption upon GR binding to the HREs. Prior to hormonal stimulation the MMTV promoter chromatin structure excluded NF1, TBP, and OTFs from their binding sites ***(6–8)***. Treatment with glucocorticoids resulted in activation of the GR, recruitment of coactivators (CoAs) and chromatin remodeling complexes (CRCs), as well as disruption of nucleosome B, subsequent binding of NF1 and OTF, and assembly of a preinitiation complex ***(9,10)***.

This chapter provides a detailed methodology for three techniques that the authors have used to analyze the chromatin structure of the MMTV promoter.

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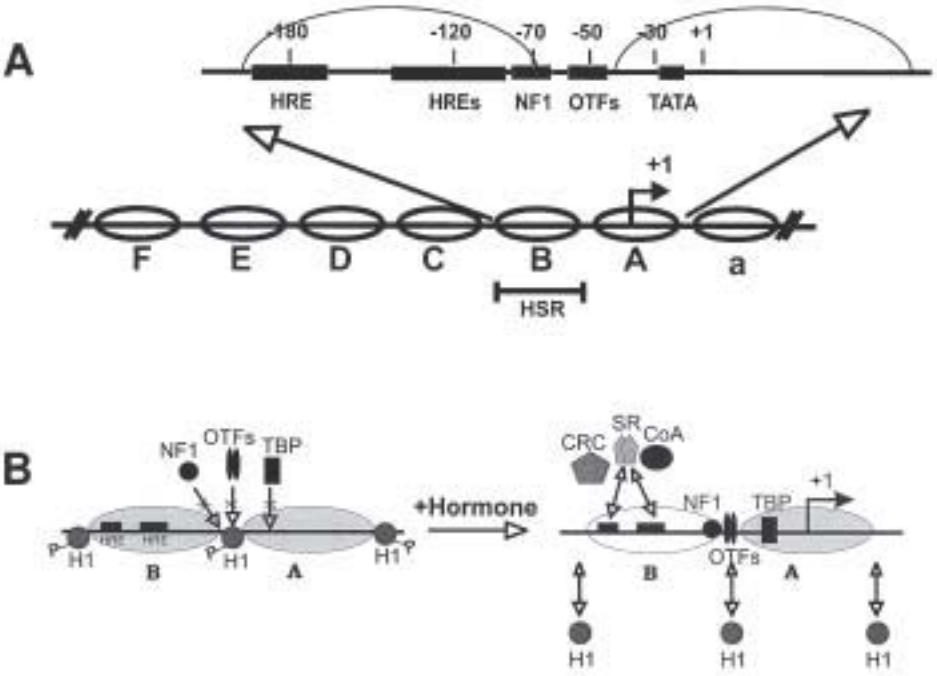

Fig. 1. **(A)** Nucleosomal organization of the MMTV promoter. When the MMTV promoter is stably introduced into cells, it is organized into a phased array of nucleosomes **(A–F)**. The hormone inducible hypersensitive region (HSR) is positioned over nucleosome B. An expansion of the region encompassed by nucleosomes A and B indicates the binding sites for important transcription factors as well as their approximate distance from the transcription start site (+1). The binding sites for the steroid receptors, hormone response elements (HREs), nuclear factor 1 (NF1), the octamer transcription factors (OTFs) and the TATA-binding protein (TBP) are illustrated. **(B)** Steroid hormones alter MMTV chromatin structure and transcription factor access. In the absence of hormone, nucleosome B is in a repressed conformation, histone H1 is phosphorylated, and transcription factors are excluded from their binding sites. Hormone treatment activates the steroid receptor, which recruits chromatin remodeling complexes (CRCs), and coactivators (CoAs), and ultimately results in a remodeling of nucleosome B to an active chromatin architecture that permits transcription factor binding and transcriptional activation.

The methodology involves isolation of intact nuclei from tissue culture cells, such that the chromatin structure can be analyzed in vivo. Although the focus is on the MMTV promoter, these techniques could be readily applied to any steroid-responsive gene to define the role of chromatin structure in its transcriptional regulation.

2. Materials

2.1. Cell Culture

Cells (*see* **Note 1**) were grown in a humidified incubator (37°C and 5% CO_2) on 150 mm tissue culture plates in 25 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 m*M* L-glutamine, 10 m*M* HEPES, 100 μg/mL of both penicillin and streptomycin, and 10% fetal bovine serum.

2.2. Nuclei Isolation Reagents (*see Note 2)

1. Homogenization buffer: 10 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, 60 m*M* KCl, 1 m*M* EDTA, 0.1 m*M* EGTA, 0.1% NP-40, 5% sucrose (sterilize through 0.45-μm filter), 0.15 m*M* spermine,* 0.5 m*M* spermidine.*
2. Wash buffer: 10 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, 60 m*M* KCl, 0.15 m*M* spermine,* 0.5 m*M* spermidine.*
3. Sucrose pad: 10 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, 60 m*M* KCl, 10% sucrose (sterilize through 0.45-μm filter), 0.15 m*M* spermine,* 0.5 m*M* spermidine.*

2.3. In Vivo Digestion by Restriction Endonucleases

1. Restriction enzyme digestion buffer: 10 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, 60 m*M* KCl, 0.1 m*M* EDTA, 5 m*M* $MgCl_2$, 5% glycerol, 1 m*M* DTT (add fresh).
2. Proteinase K buffer: 10 m*M* Tris-HCl, pH 7.6, 10 m*M* EDTA, 0.5% sodium dodecyl sulfate, 100 μg/mL proteinase K.
3. PCR stop buffer: 200 m*M* Na acetate, pH 7.0, 10 m*M* Tris-HCl, pH 7.5, 5 m*M* EDTA, 0.1 μg/μL of yeast tRNA.

2.4. In Vivo Footprinting of Transcription Factors by ExoIII

10X Mung bean buffer: 1 *M* sodium acetate, pH 5.0, 10 m*M* zinc acetate, 100 m*M* L-cysteine, 5 *M* NaCl, 50% glycerol.

3. Methods

The experimental procedure outlined below is based on studies in human and mouse mammary carcinoma cells that were stably transformed to contain multiple copies of the MMTV promoter. The use of multicopy cell lines provides a strong signal-to-noise ratio, and has enhanced the ability to define the chromatin structure of the promoter ***(11)***. The assessment of a promoter's chromatin structure by micrococcal nuclease (Mnase) digestion, restriction enzyme (RE) hypersensitivity, or ExoIII digestion is initiated by isolation of transcriptionally competent nuclei. This protocol will define the standard protocol for isolation of these nuclei, then detail the specific steps for determination of chromatin architecture by the aforementioned three methods.

3.1. Nuclei Isolation Protocol

The entire protocol is performed on ice with prechilled equipment and solutions. Cells were untreated or treated with hormone for 1 h prior to hormone

addition, then rinsed with 1X phosphate buffered saline (PBS), detached with a rubber policeman in 10 mL PBS, and transferred to a 50-mL conical centrifuge tube. Cells were pelleted in a Beckman GS-6R centrifuge at 750*g* for 5 min and PBS was removed. Homogenization buffer (5 mL) was added to cell pellet and the centrifuge tube gently swirled to dislodge the pellet. The intact pellet was transferred to a 7.5-mL Dounce homogenizer, then the pellet was gently resuspended in the homogenization buffer. After 2 min, the cells were lysed by 3–5 strokes of a Dounce A (tight) pestle (*see* **Note 3**). The lysate was transferred to a 15-mL conical centrifuge tube, and 1 mL sucrose pad was gently added directly to the bottom of the tube with a P1000 pipet. Nuclei were sedimented through this sucrose pad by centrifugation at 1400*g* for 20 min. The supernatant was gently removed and nuclei were resuspended (*see* **Note 4**) in 4 mL wash buffer and centrifuged at 750*g* for 5 min to remove traces of NP-40. The supernatant was discarded and washed nuclei kept on ice.

3.2. Micrococcal Nuclease Analysis of Chromatin Structure

Mnase is a small extracellular nuclease from *Staphylococcus aureus* that preferentially cleaves the DNA in the linker region between adjacent nucleosomes. This nuclease has been used extensively to map both the high (± 1 bp) and low (± 20 bp) resolution positions of individual nucleosomes on a target promoter (**Fig. 2**).

The analysis of chromatin organization begins with the isolation of nuclei as outlined in **Subheading 3.1.**, except that the final pellet of nuclei was resuspended in 1 mL wash buffer. Five 200-µL aliquots of nuclei were aliquoted to 6 mL polypropylene round-bottomed centrifuge tubes, and supplemented with $CaCl_2$ to a final concentration of 1 m*M*. Mnase (*see* **Note 5**) (Worthington) was added and the samples incubated at 30°C for 5 min. The reaction was stopped by adding 40 µL stop solution (100 m*M* EDTA/10 m*M* EGTA) and 1 mL proteinase K buffer. For digestion of naked DNA, 500 ng plasmid was treated with Mnase in 1 mL wash buffer containing 1 m*M* $CaCl_2$ and 60 µg/mL yeast tRNA. After 5 min at 30°C, the reaction was stopped as above. The samples were digested at 37°C for 4–24 h and then the DNA was purified by 4–6 rounds of extractions with phenol/chloroform/isoamyl alcohol (PCIA), one extraction with chloroform and precipitated by the addition of one-tenth vol 1 *M* NaCl and 3 vol 95% ethanol. After centrifugation, washing with 70% ethanol and brief drying, the DNA was resuspended in water. The samples were then treated with RNase A (100 µg/mL) for 1 h at 47°C and extracted again, once with PCIA, once with chloroform and ethanol precipitated.

DNA purified from Mnase-treated chromatin should initially be analyzed by agarose gel electrophoresis to ensure that the chromatin was cleaved by the

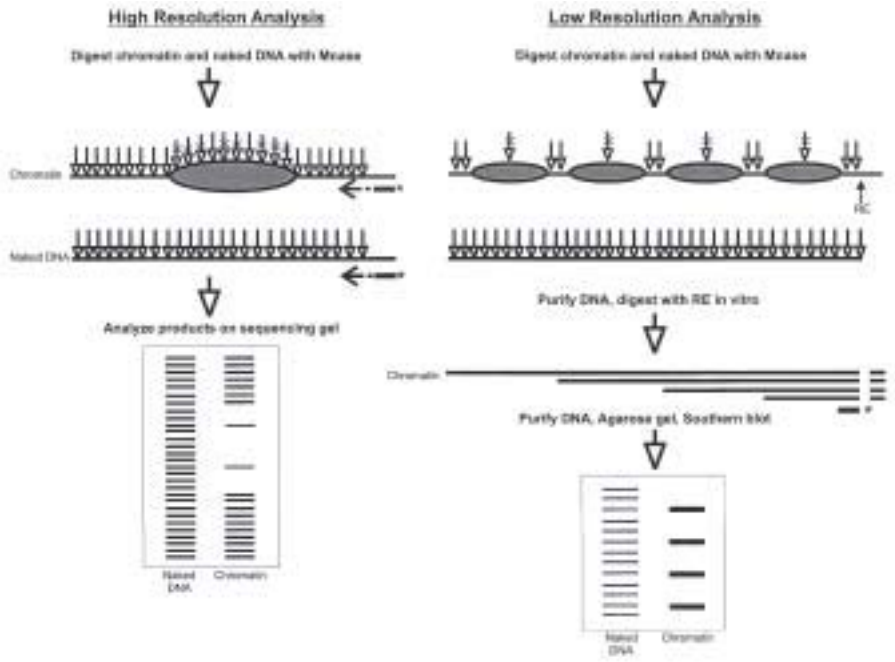

Fig. 2. High- and low-resolution analysis of nucleosome positioning with micrococcal nuclease. Isolate nuclei and digest the nuclei and naked DNA with a range of concentrations of Mnase (open arrow). For low-resolution analysis, the DNA is further digested to completion with a restriction enzyme (RE), purified, electrophoresed on 1–2% agarose gels, transferred to nitrocellulose, and hybridized with nick-translated probes. Because Mnase cleaves preferentially in the linker region of a polynucleosomal array, a series of hybridizing fragments whose lengths are multiples of the nucleosome repeat length (146 bp) should be generated. The positions of linker regions between nucleosomes, relative to the indirect end-label, can then be mapped. For high-resolution analysis, the DNA is purified, then amplified with *Taq* polymerase and a ^{32}P-endlabeled oligonucleotide. The products are analyzed on a sequencing gel. Mnase should have restricted access to the DNA that is wrapped around the nucleosome, resulting in a significant decrease in cleavage sites where the nucleosome is positioned. A comparison of the chromatin and naked DNA Mnase cleavage patterns should allow one to position the nucleosome (± 1 bp).

nuclease. Two to three micrograms of DNA from each sample should be separated on a 1.5–2.0% agarose gel. When the gel is stained with ethidium bromide, a ladder of DNA fragments (multiples of the length of DNA assembled into the nucleosome) should clearly be visible. This ladder is diagnostic of eukaryotic DNA assembled into an ordered nucleosomal array.

Low-resolution analysis of chromatin organization involves separation of digested DNA by agarose gel electrophoresis and mapping of linker regions between nucleosomes using indirect end-labeling ***(12,13)***. The sites of Mnase cleavage are mapped relative to a known restriction endonuclease site. Thus, the Mnase-treated DNA must be digested to completion with a restriction endonuclease (*see* **Subheading 3.3.**). 10–20 μg digested DNA was electrophoresed on 1.5% agarose gel and the DNA transferred to nitrocellulose (Schleicher and Shuell) according to manufacturer's specifications. The membranes were then hybridized with a nick-translated probe (*see* **Note 6**). If the gene of interest is assembled into a phased array of nucleosomes, autoradiography should generate a ladder of DNA fragments indicating the positions of nucleosomes.

Nucleotide resolution of Mnase cleavage sites involves reiterative primer extension with *Taq* polymerase of the Mnase-digested DNA with a ^{32}P-labeled oligonucleotide specific for the gene of interest (*see* **Subheading 3.3.**). Following primer extension, the products were analyzed on a 7% sequencing gel alongside sequencing reactions. Indirect endlabeling and primer extension are used to determine which DNA sequences are assembled into nucleosomes (protected from Mnase cleavage), and which are located in linker DNA between nucleosomes (susceptible to Mnase cleavage) (*see* **Note 7**).

3.3. In Vivo Digestion by Restriction Endonucleases

The washed nuclei were carefully resuspended in 0.2–0.5 mL restriction enzyme (RE) digestion buffer with a Gilson pipet (**Fig. 3**). The RE (150–1000 U/mL) was added to aliquots of nuclei (50–100 μL) and digestions were at 30°C for 15 min (*see* **Note 8**). Reactions were stopped by addition of 1 mL proteinase K buffer and incubated at 37°C for 4–24 h. Total DNA was purified by 3–6 extractions with PCIA, one extraction with chloroform and precipitated by the addition of one-tenth volume of 1 *M* NaCl and 3 vol 95% ethanol. After centrifugation, washing with 70% ethanol and brief drying, the DNA was resuspended in 180 μL of water. The DNA was digested to completion with 100 U of a second restriction enzyme (according to manufacturer's recommendation) with a cleavage site upstream of the initial in vivo restriction enzyme site (*see* **Note 9**). The redigested DNA was purified by two extractions with PCIA and one with chloroform and precipitated as described above. The DNA was resuspended in water so that the concentration was 1–3 μg/μL, as assessed by UV absorbance (*see* **Note 10**). Reiterative primer extension with *Taq* polymerase was used to determine the extent of restriction enzyme hypersensitivity. 10–20 μg purified DNA was amplified in 30 μL 1X *Taq* buffer (Gibco-BRL) with 2–4 m*M* $MgCl_2$ (*see* **Note 11**), deoxynucleotides at 200 μ*M*, 3–10 × 10^6 cpm of a ^{32}P-labeled oligonucleotide, and 2.5 U *Taq* DNA polymerase. The thermocycler was programmed for an initial cycle of denaturation

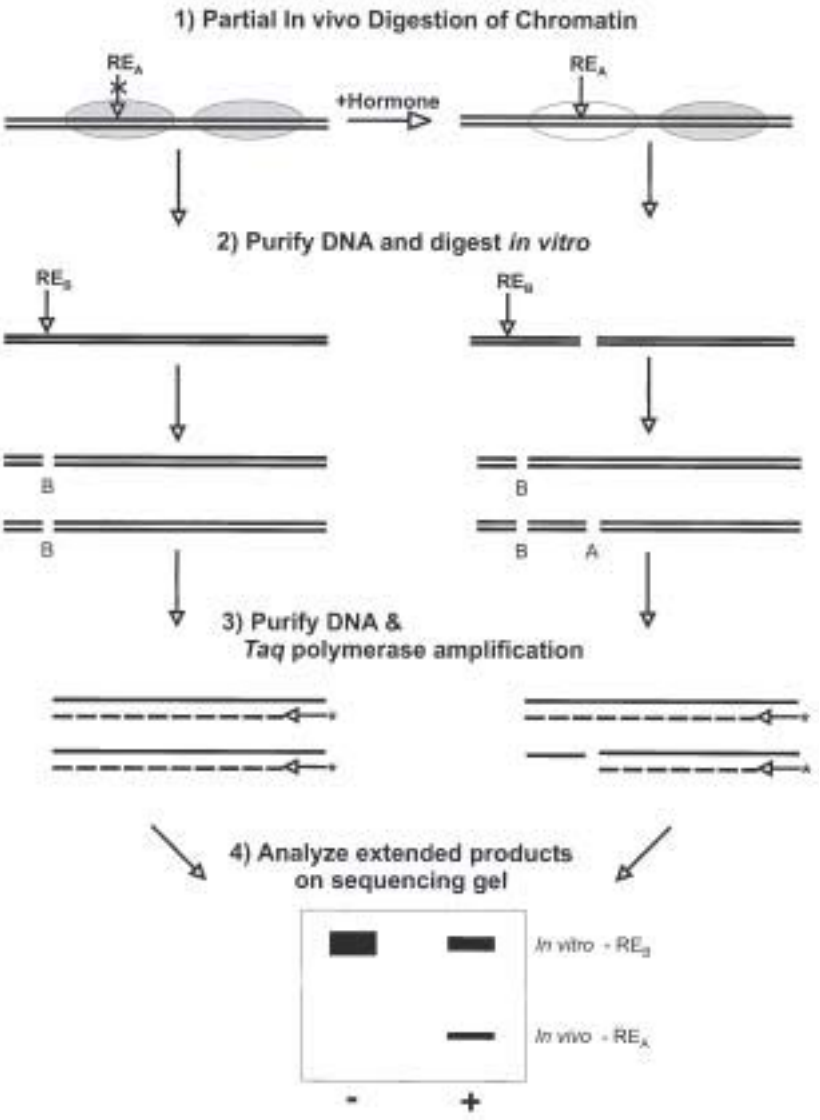

Fig. 3. Restriction enzyme hypersensitivity to detect changes in chromatin structure in vivo. Nuclei are isolated from naive and hormone-treated cells. The chromatin is partially digested with a restriction endonuclease (RE_A) that cleaves within the hypersensitive region. After purification, t he DNA is digested to completion with a second restriction enzyme (RE_B), which cleaves upstream of RE_A. Aliquots of purified DNA are analyzed by *Taq* polymerase amplification with a ^{32}P-labeled oligonucleotide primer. The amplified products are resolved on a denaturing polyacrylamide gel and products of in vitro and in vivo restriction enzyme cleavage detected by autoradiography or with the use of a PhosphorImager.

with 3 min at 94°C, 2 min at the annealing temperature of the primer, followed by 2 min at 72°C for primer extension. The additional 29 cycles were as follows: 2 min at 94°C, 2 min at the annealing temperature of the primer, and 2 min at 72°C. The final extension was for 10 min at 72°C (*see* **Note 12**). After primer extension, 100 µL stop buffer was added. The extended products were then purified by one round of PCIA, and precipitated with 2–3 vol 95% ethanol. Precipitated products were recovered by centrifugation at 4°C, dried, and resuspended in 7 µL loading buffer (80% formamide, 0.01 *M* NaOH, 1 m*M* EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol). The resuspended polymerase chain reaction (PCR) products were heated for 5 min at 94°C, and separated on 5 or 8% acrylamide sequencing gels (1X TBE). Electrophoresis should allow for maximal separation between the band corresponding to the in vitro digestion site and the in vivo restriction enzyme hypersensitivity. After electrophoresis, the gels were transferred to filter paper, dried, and exposed to Kodak X-OMAT Blue film or a PhosphorImager screen.

3.4. In Vivo Footprinting of Transcription Factors by ExoIII

ExoIII requires an entry site (*see* **Note 13**), in order to cleave DNA in a 3'-to-5' direction, until it encounters bound transcription factors (**Fig. 4**). As outlined in the **Subheading 3.3.**, the nuclei are resupended in restriction enzyme digestion buffer and entry site restriction endonuclease (300–1000 U/mL), and *Exo*III (*see* **Note 14**) (0–10,000 U/mL Gibco-BRL or NEB) were added for 15 min at 30°C. The reaction was stopped by adding 1 mL proteinase K buffer and purified as described in **Subheading 3.3.** Digestion of DNA by *Exo*III generates 5' single-strand overhangs, these must be removed prior to amplification as the oligonucleotide for primer extension binds to the undigested strand. The authors use Mung bean nuclease to digest single-strand DNA. It leaves the duplex DNA intact, and the resulting fragments represent the point at which *Exo*III digestion was impeded by bound transcription factors. Incubate purified and resuspended DNA in 1X Mung Bean buffer and 45 U Mung bean nuclease (Gibco-BRL) for 30 min at 30°C. Stop reaction by placing samples on ice and adding 5 µL 5 *M* NaCl. The DNA was further purified with two rounds of PCIA and one round of chloroform and precipitated in 2–3 vol 95% ethanol. The DNA was pelleted by centrifugation at 4°C, washed with 70% ethanol, dried briefly, and resuspended in 200 µL water. As with restriction enzyme hypersensitivity described above, the authors redigest the DNA with an enzyme that is 5' to the initial entry site (*see* **Note 15**). The purification, PCR analysis, and electrophoresis of sample is the same as described in **Subheading 3.3.** Analysis of data should include comparison of digestions that lacked either entry site enzyme or *Exo*III as well as primer extensions with plasmid and genomic DNA. This allows one to deter-

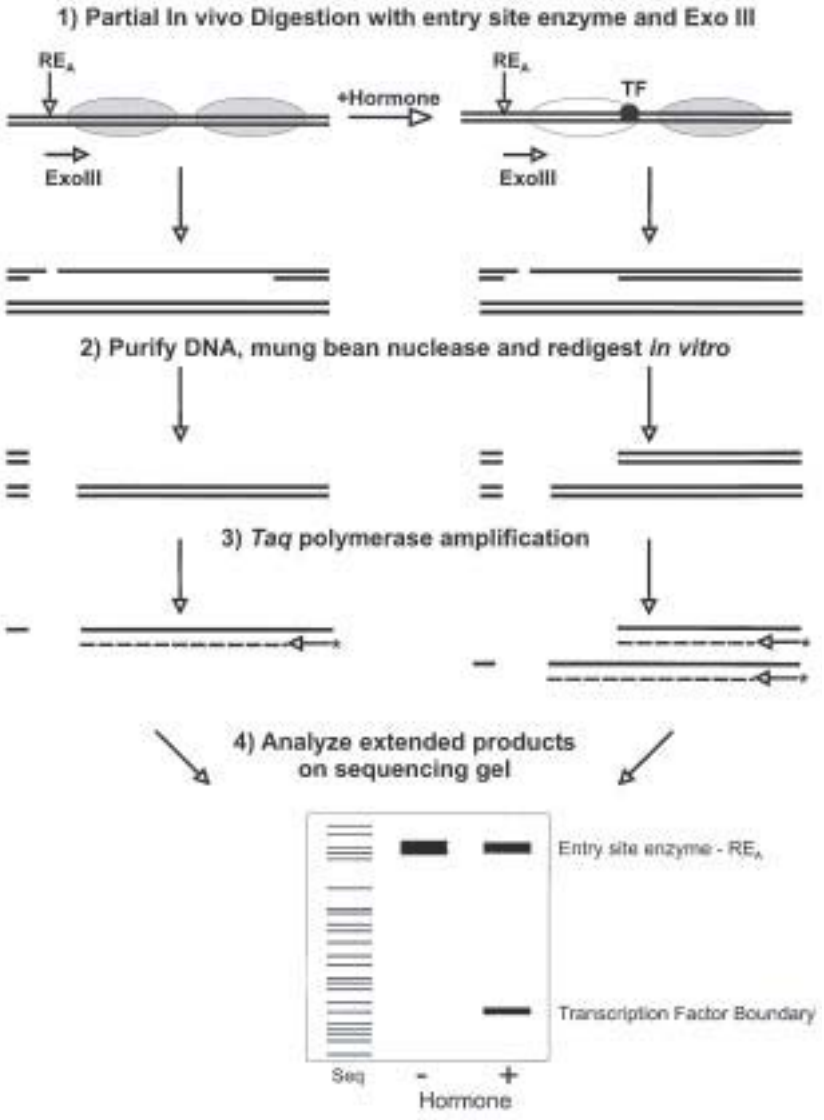

Fig. 4. Exonuclease footprinting procedure to detect transcription factor binding to chromatin. Nuclei are isolated from naive and hormone-treated cells and the chromatin digested with a restriction enzyme that provides an entry site for *Exo*III, which digests the DNA until the boundary of a transcription factor is detected. The DNA is then purified and single-strand regions removed by Mung bean nuclease. Following redigestion with a restriction enzyme in vitro, aliquots of the purified DNA is analyzed by *Taq* polymerase amplification with a ^{32}P-labeled oligonucleotide primer. The amplified products are resolved on a sequencing gel, and the transcription factor boundary detected as the appearance of a *Exo*III-dependent fragment.

mine whether the detected stops are *Exo*III dependent, and not simply the result of sequence specific pausing by *Taq* DNA polymerase.

3.5. Summary

The authors have described three approaches for analyzing the chromatin architecture of a steroid-responsive promoter. Mnase allows one to map the positions of nucleosomes on the target gene. The more sensitive restriction enzyme hypersensitivity procedure permits detection of changes in chromatin architecture upon hormonal stimulation. Additional insight into transcriptional regulation of a gene can be obtained by using the related *Exo*III footprinting protocol, which provides complementary data on transcription factor binding to chromatin templates. The use of these in vivo chromatin analysis techniques have provided evidence for a role of chromatin structure in regulation of transcription of steroid-responsive promoters including MMTV ***(2,7,10,14)***, tyrosine aminotransferase ***(15)***, TRβA ***(16–19)***, and retinoic acid receptor β (RARβ) ***(20)***.

4. Notes

1. These general conditions are for mouse C127 cells. Specific growth requirements may depend on cell type. The only requirement is that the promoter of interest responds to the hormone under the specific growth conditions.
2. All solutions are stored at 4°C and aliquoted and kept on ice during the protocol. Where indicated by *, the aliquoted solutions are supplemented with spermine and spermidine just prior to initiation of protocol.
3. The number of strokes with the pestle depends on the cell line used, however, the degree to which nuclei have been isolated from the cytoplasm can be assessed under the light microscope.
4. The nuclei should resuspend easily in the wash buffer. If the nuclei are viscous and difficult to resuspend they may have lysed to release genomic DNA.
5. Concentration of Mnase. The optimal amounts of Mnase must be empirically determined for each system, but 0–20 U/tube is a good starting point.
6. Probe for low-resolution analysis of chromatin structure by Mnase. The nick-translated probes used to analyze chromatin digestion patterns are fragments of plasmid DNA containing the promoter of the gene of interest. The restriction enzyme used to generate the 5' end of the probe should be the same restriction enzyme that was used to digest the DNA to completion.
7. Mnase analysis of chromatin structure. Mnase has been used extensively to analyze the chromatin structure of such genes as the MMTV promoter ***(2,4,21,22)*** and HIV-1 ***(23)*** in mammalian cells; the GAL80 ***(24)***, PHO5 ***(25)*** and ADH2 ***(26)*** promoters in yeast, and the Drosophila hsp26 promoter ***(27)***. Analysis of the MMTV promoter did not reveal any differences in chromatin structure upon

comparison of Mnase cleavage patterns for the closed nucleosome and the open nucleosome generated upon hormone treatment and transcriptional activation ***(2,22)***. Therefore, although Mnase may be used to determine nucleosome positioning it lacks the sensitivity of restriction endonucleases (*see* **Subheading 3.3.**) to detect changes in chromatin structure that occur upon hormone activation of the MMTV promoter.

8. Quantity and selection of restriction enzyme(s). The choice of restriction enzyme depends on the availability of sites within the promoter. Most analyses will test a set of enzymes with cleavage sites extending along the full length of the promoter. If transcription factor binding sites on the promoter are known or predicted, one may want to choose enzymes that cleave adjacent to these sites. The restriction enzyme buffer described is a generic buffer that works well with the majority of enzymes, but one may want to aliquot the nuclei prior to the wash step, so that the nuclei may be resuspended in specific digestion buffers for each individual enzyme to be tested (i.e., Gibco React 3 for *Bam*HI). The amount of restriction enzyme required will depend on efficiency of cleavage in the digestion buffer and will need to be titrated to determine the optimal concentration for maximal restriction enzyme hypersensitivity. Because this is a partial digestion of the chromatin with the in vivo restriction endonuclease, one may also want to vary the temperature of digestion from 30 to 37°C and the time of digestion in order to optimize restriction enzyme hypersensitivity between the untreated and hormone-treated nuclei.
9. Redigestion enzyme. This redigestion serves as an internal standard to ensure equal loading of DNA into the *Taq* polymerase reiterative primer extension assay and also decreases the viscosity of the genomic DNA.
10. Quantitation of DNA concentration. To ensure that the concentration of DNA determined by UV absorbance is accurate and that the DNA is homogeneously resuspended, 1–3 μg of DNA may be run on a 1% agarose gel.
11. Mg concentration and oligo selection for reiterative primer extension. As with most PCR-based reactions, the Mg concentration influences the specificity and yield of reactions, therefore, the amount of Mg will need to be titrated ***(28)***. The oligonucleotide for primer extension should bind downstream of the in vivo restriction enzyme hypersensitivity site, be greater than 18 bases long and have a T_m of 45–70°C.
12. PCR conditions. These PCR conditions have been optimized for MMTV, but will provide a good starting point for analyzing other steroid-responsive promoters.
13. Selection of entry-site enzyme. The choice of entry-site enzyme must be experimentally determined, and is typically chosen based on the positions of nucleosomes and the uniqueness of cleavage sites within linker regions. It is important to chose an entry site enzyme whose cleavage will be unaffected by the activating or repressing signals applied to cells (e.g., hormone). For instance, for the MMTV promoter, the extent of cleavage by *Hae*III is unaffected by hormone treatment and it is therefore often used as an entry site enzyme.

14. Concentration of *Exo*III. The working concentration of *Exo*III must be experimentally determined and will depend on the affinity of transcription factors for their sites on chromatin. It will also be important to conduct control experiments with the entry site enzyme alone or *Exo*III with no entry site enzyme, to determine if there are any endogenous pause sites for exonuclease on the DNA sequence being analyzed.
15. In vitro redigestion. The DNA could also be redigested to completion with the entry site enzyme, although this does not allow one to assess the extent of in vivo cleavage by the entry site enzyme.

Acknowledgments

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20

Cotransfection Assays and Steroid Receptor Biology

Shimin Zhang and Mark Danielsen

1. Introduction

The glucocorticoid, mineralocorticoid, progesterone, androgen, estrogen α and estrogen β receptors (GR, MR, PR, AR, ERα, and ERβ, respectively) form the steroid receptor family, part of the nuclear receptor superfamily ***(1)***. Like other nuclear receptors, steroid receptors have a conserved domain structure that consists of a C-terminal hormone-binding domain, a central DNA-binding domain, and an N-terminal transcriptional modulatory domain ***(2)***. However, unlike other nuclear receptors, in the absence of hormone they are associated with chaperone proteins such as HSP90 ***(3)***. Upon binding of steroid, these receptors undergo a conformational change that brings about dissociation of the receptor–chaperone complex, which in turn allows the receptor to bind to DNA, interact with transcriptional coactivators, and activate transcription ***(4)***.

This chapter discusses the use of transfection assays to analyze the transcriptional activity and hormone-binding properties of steroid receptors. Such assays are useful for the analysis of potential ligands for steroid receptors, for the characterization of mutant receptors, for the analysis of hormone-inducible promoters, and for studying the interaction of other signaling pathways on receptor activity. A typical assay system for determining the hormone-binding properties and the transcriptional activity of steroid receptors is shown in **Fig. 1**.

1.1. Expression of Receptor

In all assays described in this chapter, receptor must be expressed in cells at a level sufficient to give a clear signal. Perhaps the most common way of doing this is to overexpress receptor using transient transfection of cells with an expression plasmid. In the protocol described below, the monkey kidney-derived

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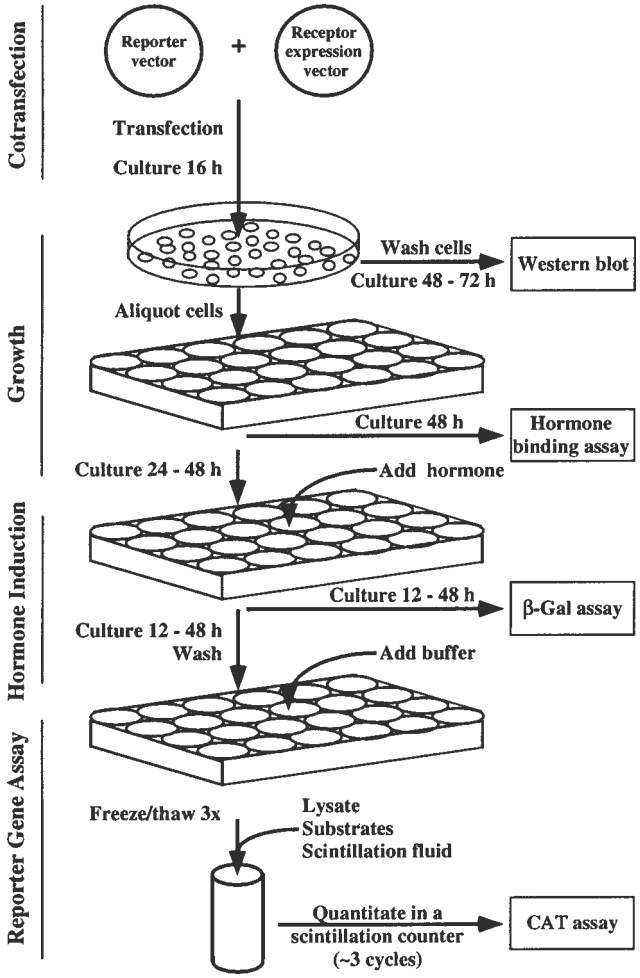

Fig. 1. Nuclear receptor transfection assays.

cell line, COS-7, is used because it does not contain steroid receptors, it is easily transfected, and the expression of SV40 T-antigen by this cell line allows replication of plasmids containing a SV40 origin of replication. *See* **Table 1** for commonly used cell lines.

An alternative to transient transfection is to stably transfect cells with a receptor expression vector and a selection vector (*see* **Subheading 3.2.**). This is particularly useful when a large number of experiments are planned for wild type receptor or a small number of mutant receptors. However, the technique is time-consuming when a large number of mutants are to be analyzed. Indeed,

Table 1
Representative Reporters and Cell Lines

Steroid receptor	Reporter vectors[a]	Cell lines[a]
GR	pMMTVCAT ***(27)***; pGRE5CAT ***(36)***; pMMTVLuc ***(37)***. pMMTV-LacZ ***(38)***; AGP-globin ***(39)***; AGP-CAT ***(39)***.	COS-7 ***(27)***; CV1 ***(25)***; CHO[b] ***(24,40)***; Ltk– ***(38)***; E8.2.A3[c] ***(5,37)***; 29+ ***(37)***; Saos-2 ***(25)***.
AR	pMMTV-CAT ***(18,25)***; pMMTV-LUC ***(41)***; C'Δ9tkCAT[d] ***(18)***; pGRE5CAT ***(25)***; pARE$_2$TK-CAT ***(42)***; probasin-CAT ***(17)***; PSA-LUC ***(41)***.	CV-1 ***(25)***; E8.2.A3 COS-7 for hormone binding assays and Western blots only ***(25)***; PC-3 cells ***(41)***.
PR	pMMTV-CAT ***(43)***; pPRE-TK-Luc ***(44)***; pPRE2-CAT ***(45)***.	CV-1 ***(46)***; HeLa ***(36,45)***; T47D[e] ***(43)***.
MR	pifG-CAT ***(47)***; pMMTV-Luc ***(48)***; pMMTV-CAT ***(49)***.	COS-7 ***(50)***; CV-1 ***(48)***; HeLa ***(47)***.
ER	pVit-TK-CAT ***(51)***; pERE-TK-Luc ***(52)***; pEREPBLCAT ***(53)***; pATC-2, ERE-vit-CAT ***(54)***.	CV1 ***(55)***; NIH3T3 ***(53)***; CHO ***(54)***; HeLa ***(56)***; MCF-7[e] ***(51)***; T47D[e] ***(51)***.

[a]The list of vectors and cell lines is not meant to be definitive, only selected vectors and cell lines are shown.
[b]Contains low levels of GR.
[c]Contains high levels of AR.
[d]Tissue-specific AR reporter.
[e]Contains high levels of ER.

although attempts at making CV1 and COS-7 cells expressing steroid receptors have been made, such stably transfected cells are not widely used. One particularly useful cell line is the mouse L-cell line, L929, which contains high levels of endogenous AR and GR, but no MR or PR. Clones of this cell line have been developed that contain a mouse mammary tumor virus (MMTV) reporter and express both the AR and the GR, or just the AR ***(5,6)***.

1.2. Use of a Hormone-Inducible Reporter

The earliest studies of receptor activity used endogenous reporters, such as tyrosine aminotransferase (TAT), whose hormonal regulation was first described in liver cells by Lin and Knox in 1957 ***(7)***. TAT became a very useful reporter because of its high level of inducibility in cells that express endogenous GR ***(8,9)***. Common problems faced when using endogenous reporters include high background levels in the absence of hormone, and a limited number of cell types that express a particular reporter. Therefore, exogenous

reporter systems are most often used for steroid receptor studies. Two notable exceptions to this are the use of endogenous PR expression as a measure of ER activity in breast cancer cells ***(10)*** and AR induction of prostate-specific antigen (PSA) expression in prostate cancer cells ***(11)***.

To measure transcriptional activity of a steroid receptor using an exogenous reporter plasmid, the reporter is transfected into cells either alone, when endogenous receptor is present or cotransfected with a receptor-expression plasmid when endogenous receptor is absent. Reporter plasmids consist of a hormone-responsive promoter driving expression of a marker gene such as chloramphenicol acetyltransferase (CAT), luciferase, green fluorescent protein, or β-galactosidase ***(12–14)***. These genes are usually of nonmammalian origin, and have no endogenous background (except for β-galactosidase at low pH).

The specificity of the reporter plasmid depends on the hormone responsiveness of the promoter that drives expression of the marker gene. Unfortunately, the commonly used promoters have only limited specificity. Estrogen-responsive reporters are the most specific, and respond only to estrogens acting through ERα and ERβ. Glucocorticoid-responsive reporters usually also respond to mineralocorticoids, androgens, and progestins acting through their respective receptors. This crossreactivity results from the similar DNA binding specificity of these four receptors. Perhaps the most common promoter used for this GR class of receptor is the MMTV long-terminal repeat ***(15,16)***. It is inducible by the GR, AR, MR, and PR in a wide variety of cell lines. Androgen reporters with various degrees of specificity have been described, although this specificity can be cell-type-specific ***(17–19)***. A list of reporter genes can be found in **Table 1**.

1.3. Transfection

Efficient delivery of the receptor and reporter genes into cells is the key for the successful determination of steroid receptor transcriptional activity. Calcium phosphate, diethylaminoethyl-dextran, lipofection, electroporation, retrovirus-mediated and adenovirus-mediated transfection are among the most popular means by which genes are introduced into cells (protocols can be found in **ref. *20***). In the protocol below, the authors use a variation of the calcium phosphate transfection technique ***(21)***. The authors obtain similar results with lipid-based systems such as lipofection ***(22,23)***. In general, all of these transfection methods give similar results (*see* **Note 1**).

1.4. Choice of Cell Lines

Although there are numerous factors to consider when choosing a cell line, perhaps the two most important ones are whether the cells can be transfected

easily and whether they contain endogenous receptors. The presence of endogenous receptors can complicate the interpretation of the results since few steroids are fully receptor specific. For instance, progesterone can activate both the PR and the GR ***(24)***. COS-7 and CV-1 are two cell lines that are often used for steroid receptor transfection experiments because they are easily transfected and because they do not contain endogenous receptors. In many cases, however, the requirement to use a specific type of cell, e.g., breast cancer cells, leads to the use of cells with one or more endogenous receptors. In these cases, the steroid receptor background of the cell line should be investigated before the cells are used (*see* **Note 2**). Care should be exercised when using virally immortalized cells since some viral oncogenes can inhibit steroid receptor activity. For instance, SV40 T-antigen inhibits the AR ***(25)*** and E1a inhibits the ER ***(26)***. Thus, COS-7 cells should not be used for AR transcription assays although they can be used for hormone binding assays and Western blots. CV1 or E8.2 cells are a good alternative for use with the AR. *See* **Table 1** for other commonly used cell lines.

1.5. Overview

This chapter presents a detailed protocol for the analysis of the GR using transient cotransfection of COS-7 cells and stable transfection of E8.2.A3 L-cells. Four basic methods are described, a simplified CAT assay, a β-galactosidase assay, a whole cell hormone binding assay, and a Western blot. The basic principles employed in these methods are applicable to other steroid receptors as detailed in the Notes.

2. Materials

2.1. Plasmids

1. pmGR is a GR expression vector that was derived from pSV2Wrec ***(27)***. It contains the mouse GR gene under the transcriptional control of the SV40 early promoter.
2. pMMTV-CAT is a GR reporter vector. It contains the bacterial CAT gene and was made by inserting the MMTV long-terminal repeat into the *Hin*dIII site of pSVOCAT ***(27)***.
3. pBAG is a β-galactosidase expression vector under the control of the Moloney murine leukemia virus long terminal repeat ***(28)***. The expression of β-galactosidase is used to monitor transfection efficiency.
4. pSV2neo contains a neomycin resistance gene driven by the SV40 early promoter ***(29)*** and is used as a selection vector in stable transfections.

These vectors should be prepared by cesium chloride density centrifugation ***(30)*** (*see* **Note 3**).

2.2. Cell Culture

1. COS-7 cells are available from the American Type Culture Collection (ATCC no. CRL-1651) (*see* **Note 4**).
2. E8.2 A3 is a GR-negative, AR-positive cell line that was derived from the GR-positive, AR-positive L929 mouse L cell fibroblast cell line ***(5)***.
3. Cell growth medium: Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) containing 10% (for COS-7 cells) or 5% (for L-cells; *see* **Note 5**) bovine calf serum or charcoal stripped bovine calf serum (CCS) (*see* **Note 6**).
4. Selection medium: 400 μg/mL G418 (Geneticin) in DMEM containing 5% serum) (*see* **Note 7**).
5. Activated dextran-coated charcoal (*see* **Note 8**). Suspend 25 g activated charcoal (Sigma) in 1000 mL distilled water; stir for 5 min. Allow the suspension to settle out on the bench for 30 min. Remove floating and suspended charcoal by removing the supernatant. Resuspend the charcoal as above and repeat the washing step twice. Resuspend the charcoal in 1000 mL 0.01 *M* Tris-HCl, pH 10.7, containing 2.5 g dextran (mol wt > 200,000, Sigma or Pharmacia), and stir for 1 h at 25°C. Store at 4°C overnight. Collect the dextran coated charcoal (DCC) by centrifugation at 800*g* for 15 min. The charcoal can be stored at 4°C for at least 1 yr.
6. Charcoal stripped bovine calf serum (CCS) (*see* **Notes 6** and **9**). Treat 2500 mL bovine calf serum with 5000 U sulfatase for 2 h at 37°C. Resuspend the DCC (from **Subheading 2.2.5.**) in the sulfatase-treated serum. Incubate the suspension in a 56°C water bath with shaking for 30 min. Remove the charcoal by centrifugation at 10,000*g* for 30 min. Sterilize the stripped serum by filtration using a 0.45-μm filter with two prefilters (*see* **Note 10**).
7. 3% and 10% CO_2 incubators.
8. General tissue culture reagents that are required include phosphate-buffered isotonic saline without calcium and magnesium (PBS); 0.05% trypsin, 0.02% EDTA in Hanks' balanced salt; and tissue culture (TC) plasticware, including 10- and 15-cm dishes, 24-well plates, and 0.45-μm filtration devices.

2.3. Transfection-Related Reagents

1. 2X BES-buffered saline (BBS) buffer; 59 m*M* N-, N-*bis*(2 hydroxy-ethyl)-2 aminoethane-sulfonic acid (BES), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, pH 7.05 (adjust the pH, using 4 *M* NaOH). The solution is stable at –20°C for at least 2 yr (*see* **Note 11**).
2. Carrier DNA (*see* **Note 12**): Dissolve salmon sperm DNA (Sigma) in distilled water about 2 mg/mL. Break DNA into short fragments (mol wt 500 bp to 50 kb) by sonication in an ice-water bath. Sterilize the solution by filtration and store at –20°C.
3. 1 *M* $CaCl_2$ and dH_2O sterilized by filtration (0.2 μm).

2.4. β-Galactosidase Assays

1. Solutions for cytochemistry:
   a. Fixing solution: 0.2% glutaraldehyde (commercial glutaraldehyde solution is 25%), 5.4% formalin in PBS.

b. X-Gal staining solution: 1 mg/mL X-Gal in 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, 2 m*M* $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate in PBS.

2. Substrate solutions for biochemical determination: 80 m*M* sodium phosphate, pH 7.3, containing 9 m*M* $MgCl_2$, 102 m*M* 2-mercaptoethanol, and either 8 m*M* *O*-nitrophenyl-β-D-galactopyranoside (ONPG, from Sigma) or chlorophenol red β-D-galactopyranoside (CPRG) (Boehringer Mannheim GmbH).

2.5. Steroid Induction and CAT Assay

1. Dexamethasone, cortisol, and triamcinolone acetonide (TA) are available from Sigma. All steroid stock solutions (10^{-3} *M*) are prepared with 95% ethanol, stored at –20°C, and diluted with cell growth medium before use.
2. 0.25 *M* Tris-HCl, pH 7.8.
3. 2 mg/mL Chloramphenicol in 0.25 *M* Tris-HCl, pH 7.8.
4. [^{3}H]acetyl-coenzyme A (CoA) with a specific activity greater than 1 Ci/mmol (1–10 Ci/mmol) is available from NEN, Boston, MA, or ICN, Irvine, CA.
5. CAT assay reaction mixture: For each reaction, mix 128 μL 0.25 *M* Tris-HCl, pH 7.8, 20 μL 2 mg/mL chloramphenicol and 2 μL [^{3}H] acetyl-CoA for a total volume of 150 μL.
6. Econofluor-2 scintillation fluid (*see* **Note 13**).

2.6. Hormone-Binding Assay Reagents

1. 10^{-2} *M* TA in 95% ethanol. 10X TA working solution should be freshly prepared just before use by diluting the stock solution with serum-free DMEM medium.
2. [6,7-^{3}H(N)]-triamcinolone acetonide ([^{3}H]-TA) with a specific activity of 30–50 Ci/mmol is available from NEN. Concentrations of 10X [^{3}H]-TA working solutions range from 10^{-11} to 10^{-6} *M* and are prepared by diluting this stock solution with serum-free DMEM medium.
3. Vehicle: 1% ethanol in serum-free DMEM medium.
4. Cell lysis buffer: 10 m*M* Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol.

2.7. Materials for Western Blots

1. Cell lysis buffer: PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 μg/mL leupeptin, 1 m*M* EDTA, 1 μg/mL pepstatin, and 0.2 m*M* phenylmethylsulfonyl fluoride.
2. DC protein assay kit from Bio-Rad, Hercules, CA.
3. Prestained high-molecular-weight markers from Gibco-BRL Life Technologies, Gaithersburg, MD.
4. ECL detection kits and peroxidase-conjugated goat antimouse antibodies are available from several companies including Amersham and KPL.
5. SDS-polyacrylamide get electrophoresis (PAGE) and Western transfer system, such as the Bio-Rad Mini Protean II system from Bio-Rad.

6. 12% SDS-PAGE gel and buffer system are prepared according to Laemmli ***(31)***.
7. 5X Protein-loading buffer: 6% SDS, 0.24 *M* dithiothreitol (DTT), 0.006% pyronin Y or 0.02% bromophenol blue, and 20% sucrose.
8. Nitrocellulose membranes from Schleicher & Schuell, Keene, NH.
9. Transfer buffer: 48 m*M* Tris, 39 m*M* glycine, 20% methanol, and 0.0375% SDS.
10. Membrane wash solution (TBS-T): 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl containing 0.05% Tween-20.
11. Blocking solution: 5% nonfat dry milk in TBS-T.
12. Anti-mGR antibody BuGR2 (Affinity BioReagents, Neshanic Station, NJ) diluted with either the wash or blocking solution as recommended by the manufacturer.

3. Methods

3.1. Cell Culture

1. Culture cells as a monolayer at 37°C in an air incubator with 10% CO_2 in 10- or 15-cm TC dishes. Each dish contains 10 mL (10-cm TC dishes) or 30 mL (15-cm TC dishes) DMEM supplemented with 10% (for COS-7 cells) or 5% (for L cells) BCS (*see* **Note 5**).
2. Feed cells every 3–4 d. Detach the cells using trypsin–EDTA solution, and transfer cells (~2×10^5 cells/10-cm dish) into new dishes (~1:10 splitting ratio) whenever cells become confluent.

3.2. Transient Transfection of COS-7 Cells with Reporter and Receptor Expression Vector

The following transfection protocol is designed for transient transfection using 10-cm TC dishes. The cell number and transfection reagents used for three different sizes of TC dishes are listed in **Table 2** (*see* **Note 14**). The results of a typical cotransfection using pmGR and pMMTV-CAT are shown in **Fig. 2**.

1. Transfer COS-7 cells to fresh 10-cm TC dishes (10^6 cells in 10 mL growth medium/dish) The cells should be about 80% confluent when they become attached. Culture the cells at 37°C in a 10% CO_2 incubator overnight.
2. Four hours prior to transfection, remove the medium, and add 15 mL fresh growth medium to each dish; continue to culture at 37°C.
3. Prepare the calcium phosphate–DNA mixture in a 15- or 50-mL sterile tube (values shown are for one 10-cm TC dish, **Table 2**).
   a. Add 1.0 μg pmGR, 10.5 μg pMMTV-CAT, 1 μg pBAG, and 17.5 μg carrier DNA (i.e., 30 μg total DNA).
   b. Add sterile H_2O to bring the volume to 562.5 μL.
   c. Add 187.5 μL 1 *M* $CaCl_2$ (do not mix).
   d. Take up 750 μL 2X BBS buffer (37°C) into a pipet attached to a pipetaid. Gently insert the pipet into the bottom of the tube. Slowly release the buffer and, after delivering the buffer, blow in about five bubbles with the pipetor to achieve a gentle mixing of the ingredients.

Table 2
Ingredients Required for the Calcium Phosphate Transfection Protocol

	Size of dish		
	6-cm	10-cm	15-cm
Cells/dish	3×10^5	1×10^6	2.5×10^6
Growth medium (mL)	5	15	30
Tranfection mixture (mL)	0.5	1.5	4.0
pmGR (ng)	5–1000	15–3000	40–10,000
pMMTV-CAT (ng)	3500	10,500	35,000
pBAG (ng)	300	1000	3000
Total DNA(μg) (use carrier DNA)	10[a]	30[a]	100[a]
1 *M* $CaCl_2$ (μL)	62.5	187.5	500
2X BBS (μL)	250	750	2000
dH_2O to final volume (mL)	0.5[b]	1.5[b]	4.0[b]

[a]Use carrier DNA to bring the total amount of DNA in the mixture to this amount.
[b]Use dH_2O to bring the final volume to this amount.

   e. To form precipitates, put the mixture in a 37°C incubator or leave it in the hood for 20 min (*see* **Note 15**).
   f. Mix the transfection mixture by pipeting up and down once.
5. Add the mixture to the 10 cm TC dish dropwise with gentle swirling.
6. Incubate the cells overnight at 37°C in a 3% CO_2 incubator (do not use a 5 or 10% CO_2 incubator).
7. Remove the medium from the dish by suction and add 10 mL prewarmed PBS. Leave PBS on the cells for 2–5 min. Gently swirl the dish several times during this period to remove precipitates. Remove PBS by suction.
8. Wash the cells again with PBS.
9. Incubate the cells at 37°C with 1.5 mL trypsin–EDTA solution.
10. Suspend the detached cells in growth medium. If multiple plates have been transfected, pool, and mix the cells together.
11. Transfer the cells to 24-well TC plates. Use one 24-well plate for each 100-cm TC dish. Each well should contain 0.5 mL growth medium.
12. Incubate the plates at 37°C in a 10% CO_2 incubator until required.

3.3. Hormone Induction of Transcriptional Activity

1. Cells can be treated with hormone as soon as they have reattached to the plate or they can be treated the next day.
2. Add 0.5 mL growth medium containing 2X the final concentration of steroid required (*see* **Note 16**) into each well to be induced with hormone for a final volume of 1 mL. Add 0.5 mL growth medium containing 1% ethanol to each control well. Inductions and controls should be performed in triplicate.
3. Incubate the plates at 37°C in a 10% CO_2 incubator for 12–48 h.

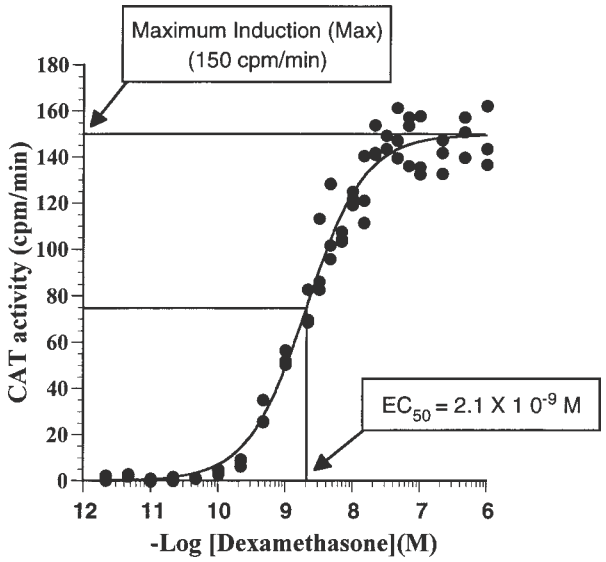

Fig. 2. GR dose–response curve. COS-7 cells were cotransfected with 1 μg/dish pmGR and 10.5 μg/dish pMMTV-CAT by the calcium phosphate method as detailed in **Subheading 3.2.** The cells were treated 24 h later with the indicated concentrations of dexamethasone (DEX) (in triplicate, **Subheading 3.3.**). CAT activity was determined 2 d later. The dose–response curve was computer-fit using the software program DeltaGraph (SPSS, Chicago, IL) and the relationship:

$$CAT = [DEX] \times Max / ([DEX] + EC_{50})$$

where CAT = CAT activity in cpm/min, Max = the theoretical maximum CAT activity obtainable, and EC_{50} is the concentration of steroid that gives half maximal induction (*see* **Note 28**). Computed values were $EC_{50} = 2.06 \times 10^{-9}$ *M DEX*, Max = 149.7 cpm/min; $r^2 = 0.98$.

3.4. Determination of CAT Activity

1. Remove the medium from the steroid-treated cells by inverting the multiwell plate. Wash the cells twice with PBS. Remove the residual PBS from the plates by leaving the plates upside-down on paper towels for 1 min or use suction.
2. Add 250 μL of 0.25 *M* Tris-HCl, pH 7.8 to each well.
3. Freeze and thaw the plates 3× at –70°C and room temperature. Mix the lysates by swirling the plates several times after each thaw.
4. If required, inactivate deacetylases by incubating the plates at 65°C for 10 min (*see* **Note 17**).
5. Transfer 100 μL cell lysate into a 6-mL scintillation vial.
6. Add 150 μL CAT assay reaction mixture. Mix by briefly vortexing.

7. Add 2 mL Econoflor-2 to each vial. Vortex for 2 s.
8. Transfer the vials into a scintillation counter. Count samples for 30 s for a total of 3 cycles.

3.5. β-Galactosidase Solution Assay (32)

1. Prepare lysates as in **Subheading 3.4.** but do not heat-inactivate.
2. Mix 800 μL of the substrate solution with 200 μL lysate.
3. Incubate the mixture in a 37°C water bath for 2 h (CPRG) or overnight (ONPG).
4. Measure the absorbance at 570 nm (for CPRG) or 414 nm (for ONPG) using a spectrophotometer. Controls should include lysates of cells transfected without the pBAG vector.

3.6. β-Galactosidase Cytochemical Assay

1. Following transfection (**Subheading 3.2.**), distribute cells into multiwell plates.
2. Culture for 2 d.
3. Wash the cells twice with PBS.
4. Fix the cells with the 4°C cold fixing solution for 5 min, then wash the cells three times with PBS.
5. Add enough staining solution to cover the cells and incubate the cells overnight at 37°C.
6. Count the blue-stained cells under an inverted microscope.

3.7. Analysis of CAT Assay Data

The rate of the CAT reaction is determined by calculating the number of cpm produced per minute. The simplest method is to use the formula (*cpm3 – cpm1)/time*, where *cpm1* refers to the first cycle in the scintillation counter, *cpm3* is the third cycle, and *time* is the time elapsed between the first and third cycles (*see* **Note 18**). Alternatively, cpm can be plotted against time and the rate determined from the slope. Within a transfection, no normalization needs to be conducted. To compare results from different transfections, CAT activities should be normalized by the results of either of the β-galactosidase assays.

3.8. Whole-Cell Hormone-Binding Assay

1. Transfect cells as in **Subheading 3.2.**, except that no pMMTV-CAT is required (*see* **Note 19**).
2. Wash the transfected cells twice with prewarmed PBS the following day.
3. Detach the cells with trypsin–EDTA solution.
4. Suspend the cells in the growth medium. Distribute the suspension into 24-well plates (cells from one 10-cm dish to one plate). Each well should contain 1 mL cell suspension.
5. Culture cells at 37°C in a 10% CO_2 incubator for 2 d.

6. Wash the cells once with 37°C serum-free medium.
7. Add 80 μL serum-free medium and 10 μL 10X concentrated [^{3}H]-TA working solution to each well. To half of the wells, add 10 μL vehicle. To the other half of the wells, add 10 μL nonradioactive competitor TA. The final concentration of cold TA should be 500–1000× the concentration of [^{3}H]-TA. The concentration of [^{3}H]-TA used should be confirmed by quantitation in a scintillation counter.
8. Incubate the plates at 37°C for 2–3 h, with occasional swirling.
9. Remove the binding reaction solution from wells. Wash the cells twice with PBS.
10. Add 150 μL lysis buffer to each well and incubate at room temperature for 15 min.
11. Transfer the cell lysate into scintillation vials.
12. Add 2 mL of a scintillation fluid designed for aqueous samples (e.g., Ecoscint A, National Diagnostics, Atlanta, GA) to each vial and mix by vortexing.
13. Specific activity can be obtained by subtracting nonspecific binding from total binding. K_d can be calculated using a Scatchard plot or by curve fitting ***(30)***.

3.9. Quantitation of Receptor Levels

Receptor levels can be quantitated by two methods, hormone-binding assays (above) and Western blots. This Western blot protocol is specific for the GR, but is easily adapted for other steroid receptors.

1. Transfect cells with the GR expression vector pmGR (*see* **Subheading 3.2.** and **Note 20**).
2. Next day, wash the cells twice with prewarmed PBS, feed the cells with growth medium (10 mL/dish), and culture for 2 d at 37°C in a 10% CO_2 incubator.
3. Remove the culture medium and wash the cells once with PBS.
4. Add 1.5 mL ice-cold cell lysis buffer to each dish and incubate at 4°C for 10 min.
5. Transfer the cell lysates to microcentrifuge tubes and remove cellular debris by centrifugation at 4°C and 10,000 rpm for 15 min.
6. Determine the protein concentrations of the lysates using the Bio-Rad DC protein assay kit. If the lysates are not used immediately, store at –70°C until needed.
7. Mix the lysates with 5X loading buffer. Heat the mixture in a boiling water bath for 3 min.
8. Load the mixture onto a 12% SDS-PAGE gel (20–30 μg lysate protein/lane) (*see* **Note 21**). Separate proteins by electrophoresis.
9. Transfer the proteins from the gel to a nitrocellulose membrane using a Bio-Rad or similar transfer apparatus.
10. Block nonspecific binding by shaking the membrane in the blocking solution overnight.
11. Wash the membrane twice with the wash buffer with shaking for 10–15 min.
12. Incubate with the GR-antibody for 2 h (use the concentration recommended by the manufacturer). Wash the membrane three times with wash buffer.
13. Incubate with peroxidase-conjugated second antibody for 1 h. Wash the membrane as above.

14. Incubate the membrane in ECL solution (1:1 mixture of solution A and solution B) for 1 min.
15. Remove residual ECL solution from the membrane by putting the membrane between two pieces of filter paper (do not let it dry completely).
16. Cover in Saran wrap.
17. Expose the membrane to X-ray film for 5 s to 5 min, depending on the strength of the signal, and develop the film.

3.10. Stable Transfection of L-Cells with a Receptor-Expression Vector

1. Transfect L929 or E8.2 A3 cells as described in **Subheading 3.2.**, except that for each 10-cm dish, use 3 μg pmGR, 10.5 μg pMMTV-CAT, and 0.1 μg pSV2neo, 16.4 μg carrier DNA (*see* **Notes 22** and **23**).
2. The day after transfection, wash the cells twice with 37°C prewarmed PBS.
3. Detach the cells with trypsin–EDTA solution, add growth medium, and plate the cells in fresh plates (1:10).
4. The next day, replace the culture medium with fresh medium containing 400 μg/mL G418 (selection medium) (*see* **Note 24**). Feed the cells every 3 d with the selection medium.
5. Culture the cells at 37°C in a 10% CO_2 incubator until single colonies can be seen by eye.
6. To pick single colonies, remove the medium from the dish, take 2–5 μL trypsin–EDTA solution in a pipet tip. Touch a single colony with the tip. Detach the colony from the culture plates by pipeting up and down several times.
7. Transfer individual colonies to 24-well TC plates containing growth medium (1 colony/well).
8. Culture the cells in the selection medium until there are enough cells for screening positive colonies by CAT assays or Western blot as described above (*see* **Notes 25–27**).

4. Notes

1. In general, the receptor assays detailed herein are independent of the transfection method used. However, differences are sometimes observed. For example, highly efficient transfection techniques can lead to levels of receptor that are high enough to cause self-squelching ***(33)***.
2. The receptor content of a cell is important because some steroids can activate more than one receptor type. Highly specific steroids include testosterone, dihydrotestosterone, and R1881, which only activate the AR, and R5020 (a synthetic progestin), which only activates the PR. These receptor-specific steroids could be used to differentiate the hormone response in a multi-receptor cell system. Estradiol activates both ERα and ERβ and can have weak activity on the AR. In tissue culture systems, glucocorticoids and mineralocorticoids activate both the GR and the MR. Progesterone is a weak GR agonist. R5020 is a glucocorticoid receptor antagonist.

3. The purity of the DNA is particularly important. The authors prepare the crude DNA in DNase free RNase solution, then purify the DNA using double CsCl centrifugation. Other methods of purifying the DNA can work, but care should be taken to ensure that they give RNA-free DNA.
4. COS-7 cells were derived from the CV-1 cell line (African green monkey kidney cells) by transformation with SV40 T-antigen ***(34)***. Plasmids containing an SV40 origin of replication such as pmGR and other pSV2 derived vectors replicate to high copy number in COS-7 cells but not in CV-1 cells. COS-1 cells can also be used; these produce less SV40 T-antigen than COS-7 cells.
5. L929 derived cells will often round up and float in growth medium containing 5–10% serum. Reducing the serum concentration to 3% can prevent this.
6. For routine cell culture, use bovine calf serum. Charcoal-stripped calf serum (CCS) is only required when endogenous levels of steroid in the serum are high enough to induce the receptor of interest. For the ER, CCS (and phenol red-free growth medium) is always required. For the AR, CCS can decrease the background, especially in cells stably transfected with a reporter gene. For the GR and MR, CCS is rarely required unless the cells also contain AR.
7. Concentrations refer to the active component because some preparations are not 100% pure.
8. Dextran coated charcoal can also be purchased from Wein, Succasunna, NJ.
9. CCS can be purchased from Hyclone, Logan, Utah.
10. 0.45 μm filtered serum is fine for transient transfections in which the cells are discarded at the end of the experiment. For stable transfections and for long term growth of cells, the 0.45-μm filtration should be followed by a 0.2-μm filtration step. Direct filtration of the medium through a 0.2-μm filter can be difficult; one alternative is to add the serum to the DMEM first, and then filter.
11. The 2X BBS is the most important component of the transfection; make a large batch and freeze it. The pH of this solution should usually be exactly 7.05. To ensure similar transfection efficiencies, always use the same number of cells, and grow the cells in fresh medium before transfection. This ensures that the final pH during transfection remains constant. If necessary, the pH of 2X BBS can be titrated in increments of 0.01 pH units and its efficiency tested in transfection experiments.
12. Plasmid DNA can also be used. If the transfection protocol has been optimized for one of these DNA preparations, reoptimization will be required if the carrier DNA is changed.
13. The authors have found that only Econofluor-2 works well in this assay. In the absence of CAT activity, the initial cpm should be no more than a few hundred cpm. Over 1000 cpm indicates a problem with the scintillation fluid or possibly the [^{3}H]acetyl-coenzyme A, given a counting efficiency of ~30%.
14. The amounts of the cotransfection components listed in **Table 2** may be changed slightly. However, the following principles should be adhered to:
    a. Cells in culture should be around 80% confluent.
    b. The ratio of total volume of the transfection mixture to 2X BBS to 1 *M* CaCl2 is 8:4:1 and should not be changed.

c. To ensure excess reporter genes in the transfected cells, pMMTV-CAT should be at least 3.5× more than the receptor vector.

15. The best transfections are obtained with fine precipitates. These precipitates make the medium translucent. Coarse precipitates decrease the transfection efficiency, and, in addition, they can make it difficult to detach the cells from the plates if cells continue to grow in the same plates more than 2 d.
16. For dose-response curves (**Fig. 2**), serial dilutions of 2X concentrated steroid solutions ranging from 10^{-12} *M* to 10^{-6} *M*, should be prepared by diluting a stock solution with growth medium. Single concentration hormone-binding assays of the GR can also be performed. In transfected cells, the EC_{50} for dexamethasone is ~2×10^{-9} *M*. Thus, a near-maximum induction is given by 10^{-7} dexamethasone. For other receptors, near maximum induction can be obtained by using 10^{-9} *M* estradiol for the ER; 10^{-8} *M* dihydrotestosterone for the AR; 10^{-8} *M* aldosterone for the MR, and 10^{-8} *M* progesterone for the PR.
17. Some cells contain deacetylase that degrades the products of the CAT reaction. Incubating cell lysates in a 65°C water bath for 10 min inactivates this enzyme and increases the assay's sensitivity ***(35)***. This is not usually necessary for COS-7 cells.
18. The difference between any two cycles can be used as long as the reaction is still in the linear phase. For accurate quantitation, lysates with activity over about 100 cpm/min should be diluted and reassayed. The background (no lysate) should be no higher than 3 cpm/min.
19. For standard hormone-binding analysis of a receptor, no reporter plasmid is required. However, if both the hormone-binding properties and the transcriptional properties of the receptor are to be studied in parallel, reporter should be used. Up to 30 μg receptor expression vector can be used to ensure that there is enough receptor produced to allow accurate measurement of hormone binding activity (5 μg should be more than sufficient).
20. For standard expression analysis of a receptor, no reporter plasmid is required. However, if both the expression properties and the transcriptional properties of the receptor are to be studied in parallel, reporter should be used. Up to 30 μg receptor expression vector can be used to ensure that there is sufficient receptor produced to detect on a Western (5 μg should be sufficient).
21. SDS gels of 8–12% can be used.
22. Any transfection method can be used. However, the ratio between the receptor expression vector and the selection vector should be at least 10:1 to ensure that cells that take up the selection vector also take up the receptor expression vector. pMMTV-CAT can be reduced to the same level as the expression vector if the transfection protocol requires it.
23. Just the reporter plasmid or just the receptor-expression plasmid can be transfected into the cells if desired. L929 cells express high levels of GR and AR. E8.2 A3 cells express just the AR. L929 and E8.2 A3 cells, stably transfected with MMTV-CAT, are available from the authors.
24. The concentration of G418 required for selection of resistant cells varies from one cell line to another. To determine the optimal concentration of G418 for a

cell line, make serial dilutions of G418 stock solution with cell growth medium on a 96-well TC plate. Seed 10^4 cells into each well. Culture the cells for 7–10 d. Monitor cell growth in the cultures. The optimal concentration of G418 is the lowest concentration to cause complete cell death. Cell death can take up to 10 d.

25. To screen colonies expressing GR and pMMTV-CAT, make duplicate plates and treat one plate with TA for 24 h, and measure CAT activity.
26. To screen colonies transfected with just the receptor-expression plasmid, make duplicate plates, transiently transfect one plate with pMMTV-CAT, treat with TA, and screen for positive colonies using CAT assays. An alternative is to screen for GR expression using Western blots or to do single-point hormone-binding assays.
27. To screen colonies transfected with just pMMTV-CAT, make duplicate plates, transiently transfect one plate with the GR expression vector, treat with TA, and screen for positive colonies using CAT assays. For cells expressing endogenous AR, GR, MR, or PR, treat with the appropriate hormone directly and screen for positive colonies using CAT assays.
28. Cotransfection assays can also be used determine the antagonist activity of steroids; methods can be found in **ref. *24***.

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21

Estrogen Receptor mRNA *In Situ* Hybridization Using Microprobe System

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1. Introduction

1.1. Estrogen Receptor

Biological effects of steroids are mediated through an initial interaction with specific receptor belonging to a member of the steroid/thyroid/retinoid receptor gene superfamily ***(1,2)*** . Recently, increasing numbers of new members of this family, including their subtypes have been cloned and characterized. For instance, until the recent cloning of a second estrogen receptor (ER) α, ERβ had been considered as the only receptor able to bind estrogen with high affinity. ERα protein is smaller than ERβ protein but has a similar high affinity for estradiol, as does the receptor ***(3,4)***. Both receptors demonstrate high conservation of amino acid sequence in regions of the hormone-binding domain known to be important in contacting ligands ***(3)***. However, tissue distribution and relative expression levels of ERα have been demonstrated to be different from those of ERβ, which suggests possible different biological roles of ERα in mammalian estrogen dependent tissues ***(5)***.

1.2. Significance of Localization of Estrogen Receptor

As described above, it is important to examine the tissue distributions or *in situ* expression of these newly identified member(s) of the steroid receptor family in order to obtain a better understanding of their functions and/or other relevant biological significance. Immunohistochemistry of steroid receptor has provided important information as to its intracellular and/or intratissue localization. For instance, immunohistochemistry of estrogen and progesterone receptors has been widely used in the evaluation of hormonal therapy in patients

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with breast carcinoma. However, it is also true that immunohistochemistry requires the reliable antibody generated in mouse or rabbit or other mammals, that is not necessarily the case with newly identified family of steroid receptors. In addition, the specific antibody which recognizes the protein in immunoblotting may not be suitable for immunohistochemistry.

1.3. mRNA In Situ Hybridization of Estrogen Receptor

mRNA *in situ* hybridization can demonstrate the localization of mRNA signals in tissue sections and has been extensively utilized in various investigations ***(6)***. Especially, antibodies or the full sequences of the substances are not necessarily required if specific oligonucleotides are employed as probes of hybridization. However, it is also true that the method itself is time-consuming and laborious, which makes the technique not widely available in laboratories ***(6)***. The development of computer-assisted control of each steps of the method and specifically designed probes with sensitive labeling, made the automatic and/or semiautomatic procedure of mRNA *in situ* hybridization possible. This chapter describes mRNA *in situ* hybridization study of steroid receptor(s) using the Microprobe staining system (Fisher, Pittsburgh, PA) with manual capillary actions.

2. Materials

2.1. Fixatives

Fixatives most suitable for mRNA *in situ* hybridization study using the Microprobe staining system are as follows:

1. 4% Paraformaldehyde adjusted to pH 7.4. (4% Paraformaldehyde should be kept at 4°C and should be freshly prepared every 2–3 wk.)

or

2. 1% Glutalaldehyde in 4% paraformaldehyde adjusted to pH 7.4. Glutalaldehyde should be mixed with paraformaldehyde only prior to actual fixation and should not be stored once prepared. Glutalaldehyde solution can be frozen at –20°C.

Regular 10% neutral formalin may be used as fixatives, but these two fixatives are more preferable, at least for mRNA *in situ* hybridization using Microprobe staining system. Snap-frozen sections may be used, but crosslinking fixatives described above yielded better morphological details, which is required for interpretation of mRNA *in situ* hybridization and increased retention of small nucleic acids in tissue sections.

2.2. Glass Slides and Others

2.2.1. Glass Slides for mRNA In Situ Hybridization

Tissue adherence to glass slides is cardinal for the success of mRNA *in situ* hybridization because loss of tissue sections during the procedure of mRNA *in*

situ hybridization is a common problem, resulting in marked disappointment. Clean Fisher Probe On Plus Microscope Slides (Fisher) should be used for mounting the tissue sections. Sections of uniform thickness (3–4 μm) without folds or tears are absolutely required. These glass slides can be stored at room temperature. Any glass cover slips can be used, but they should be clean.

2.2.2. Other Materials for mRNA In Situ Hybridization

Other materials used in the Microprobe staining system including buffer and enzymes are all commercially available from Research Genetics Co. Ltd. (Huntsville, AL). Instruments for performing mRNA *in situ* hybridization including the chambers and incubators can be all purchased from Fisher as the Microprobe staining system.

2.3. Probes

Oligonucleotide probes employed for hybridization in Microprobe staining system are synthesized with a 3' biotinylated tail (Brigati tail) (5'-probe-biotin-biotin-biotin-TAG-TAG-biotin-biotin-biotin-3') or specific multibiotinylated reaction *(7)*. This labeling is presently exclusively performed at Research Genetics, but can be commercially ordered. These probes are synthesized with six biotin molecules at the 3' end, and oligomers labeled at or on the ends of the hybridization sequence gave stronger hybridization signals than oligomers with internal labels. However, oligonucleotides labeled in this manner can be synthesized only up to 30-mers in length. These sequence-specific probes of short length allows for a shorter hybridization time, compared to other methods of mRNA *in situ* hybridization. Therefore, every effort should be made to avoid any possible redundancy or crossreaction of any known DNA sequences using computer-assisted search engines when designing these short oligonucleotide probes for the Microprobe staining system. The difference of mRNA hybridization reaction is also influenced by the nucleotide composition of the probes employed. Nucleic acid with a high glucocorticoid content are usually considered to produce more stable hybrids, but the most satisfactory results are obtained when using oligonucleotide probes with 60–65% glucocorticoid content in mRNA *in situ* hybridization using the Microprobe staining system. Sense oligonucleotide probes, which are used as negative controls, should also be prepared in mRNA *in situ* hybridization using the Microprobe staining system. The mixture or cocktail of oligonucleotides with corresponding sequences of the genes enhanced the sensitivity of the reaction as in other forms of mRNA *in situ* hybridization using oligo-probes for hybridization reactions.

3. Method

3.1. Fixation of Specimens

The most important step of mRNA *in situ* hybridization is the fixation of the specimens. Without proper fixation, no reliable mRNA hybridization signals

could be obtained, even using the best available methods of mRNA *in situ* hybridization. The details of this most important step in this procedure is described in **Notes 1–4**.

3.2. Pretreatment of Tissue Slides

In situ hybridization performed with the Microprobe staining system using manual capillary actions were first reported by Montone et al. ***(8–11)***, in detecting viral gnomes in routinely processed surgical pathology materials, primarily for diagnostic purposes. The authors have utilized the methods for investigative purposes ***(12–17)***, including mRNA *in situ* hybridization analysis of ERα and β in human endometrium ***(12)*** and human breast cancer ***(14)***. The procedure of mRNA *in situ* hybridization performed with the Microprobe staining system using manual capillary actions is summarized in **Table 1**.

Briefly, tissue sections (3 μm, applied to ProbeOn Plus slides) were rapidly dewaxed, cleared with alcohol, rehydrated with a Tris-base buffer, pH 7.4 (Universal Buffer, Research Genetics, Huntsville, AL), which should be stored at 4°C. These reactions were carried out by immersing the slides in the solutions, and these solutions subsequently covered the entire tissue sections by capillary reactions. The sections were then digested with pepsin (2.5 mg/mL, Research Genetics) for 3 min at 105°C in a closed chamber box included in the Microprobe staining system. This pepsin treatment is required for all crosslinked specimens. Digestion removes proteins and makes the target more accessible to the probe. Acid conditions in the pepsin solution also contribute to tissue permeabilization and protein removal. The type of specimens and degree of crosslinking should determine this process caused by fixative employed.

3.3. Hybridization

Probe was applied in formamide-free diluents (Research Genetics) and the slides were heated to 105°C for 3 min in the closed chamber box for denaturation. Probe concentration influenced efficacy of hybridization and level of background or nonspecific interaction of probe and tissue. In addition, time of hybridization may be decreased, if probe concentration is increased. Therefore, empirical preliminary study was required for determining the most appropriate concentrations of the oligonucleotides probes employed. The probes can be stored under stable conditions at –20°C for up to 2 yr but thawing and refreezing of the probes should be avoided. Therefore, the probes should be allocated into small volumes for storage. The probe solution can be stored at 4°C for up to 2 wk. The reacted tissue sections were then cooled for approx 1 min at room temperature, and allowed to hybridize at 45°C for 60 min.

Table 1
Procedure of mRNA *In Situ* Hybridization Using the Microprobe Staining System

Reagent	Time	Temperature
Dewaxing agent	3 min	105°C
Dewaxing agent	3 min	105°C
Absolute alcohol	3 washes	RT[a]
1X Universal buffer	3 washes	RT
Pepsin solution	3 min	105°C
1X Immuno./DNA buffer	3×	RT
Probe/hybridization	3 min	105°C
Probe/hybridization	60 min	45°C
2X Standard sodium citrate	3 min (×2)	45°C
Streptavidin alkaline phosphatase (AP)	10 min	50°C
AP chromogen buffer	3×	RT
Stable Fast Red TR solution	15 min	45°C
dH_2O	3×	RT
Hematoxylin	30 s	RT
dH_2O	3×	RT
Air-dry	3 min	60°C

Mount with Pristine Mount. RT, room temperature.

3.4. Washing of the Hybridized Tissue Slides

The sections were then washed twice with 2X standard saline citrate at 45°C (3 min/wash) to remove excess probes from the tissue sections. Increasing the temperature and lowering the salt concentration may result in destabilization of hybrids with base-pair mismatch, and preliminary study may be required for determining the most suitable conditions.

3.5. Detection of Hybridized Products

The tissue sections were then detected with alkaline-phosphatase-conjugated streptavidin (Research Genetics). The colorimetric detection agents will influence the final sensitivity of the reaction. In addition, low background is required for optimal investigation. Both horseradish peroxidase systems using diaminobenzidine as a chromogen, and alkaline phosphatase systems using fast red or blue as chromogen, are available as colorimetric agents in mRNA *in situ* hybridization using the Microprobe staining system, but the latter usually yield better results. After washing once in AP Chromogen Buffer, pH 9.5 (Research

Genetics), at room temperature, hybridization products were visualized with fast red. Chromogen reaction solutions are prepared by mixture of an equal volume of Stable Fast Red TR (Research Genetics) and Stable Naphtol Phosphate. These solutions should be separately kept in dark at 4°C. These freshly mixed solutions should be immediately applied to tissue sections. The slides were counterstained with hematoxylin, air-dried, and cover-slipped for microscopic examination using Pristine Mount (Research Genetics). Fast red colorimetric reactions may be faded following reactions, and the images should be taken by photographs or captured through CCD camera.

4. Notes

1. Mode of fixation. Prompt and brief fixation is considered as one of the most important keys to the success of mRNA *in situ* hybridization, which using the system described in this chapter, is highly specific. However, sensitivity of mRNA *in situ* hybridization is determined by fixation conditions and/or tissue procurement. In cases of small laboratory animals such as rats, perfusion with fixatives can be used for fixation, which usually results in satisfactory fixation of the tissues for mRNA *in situ* hybridization. However, this mode of fixation cannot be practically applied to large laboratory animals and human and may be impossible. Therefore, fixation is performed by immersing tissue specimens into fixative. Every effort should be made to permeate tissue specimens with fixatives, regardless of the fixatives employed. Insufficient fixation results in degradation and/or diffusion of mRNA targets in the cells and distorted architecture of the tissue specimens, which results in false-negative reactions of hybridization signals, and makes interpretation of the findings impossible. Excessive fixation, e.g., using the fixative with high concentration or fixation for a long duration, also results in degradation of mRNA signals in the cells and extensive formation of crosslinking, which makes it difficult to detect hybridization signals.
2. Tissue procurement of the specimens. Initial important step is trimming of the specimens. The most appropriate thickness of the specimens may be dependent on the nature of the tissues examined, but in general a thickness of up to 5 mm allows sufficient permeation of the fixatives into tissue specimens. Sufficient volumes of fixative are necessary because expected and constant shaking of specimens in fixative employed, which may be achieved by placing the container with fixatives and specimens in the regular water bath, can facilitate the process of fixation. The tissue containing fat tissue such as breast or adrenal gland floats in the fixative, which prevents fixation on the surface of the specimens. In this case, tissue paper or other appropriate paper materials should be placed on the surface of the specimens in order to avoid drying of the tissue. Microwave irradiation may be used in order to facilitate the process of fixation but is by no means recommended in all cases. When the fixation is performed in the manner above, the tissue specimens may be satisfactorily fixed for mRNA *in situ* hybridization

for 12–24 h at 4°C when using 1% glutalaldehyde in 4% paraformaldehyde adjusted to pH 7.4 as a fixative, and for 18–36 h at 4°C when using 4% paraformaldehyde adjusted to pH 7.4 alone as a fixative for mRNA *in situ* hybridization.

3. Rapid procedure of mRNA *in situ* hybridization. mRNA *in situ* hybridization using the Microprobe staining system and multibiotinylated oligoprobes can be finished within 4 h, including preparation of the materials used for the procedure. However, incubation time of the reactions and interval between the steps are relatively brief and all materials required for each step of the reaction should be prepared in advance throughout the procedure. While performing this procedure, no other laboratory works, should be performed in parallel and empirical concentration on the procedure is required much more than established methods of mRNA *in situ* hybridization.
4. Control of mRNA *in situ* hybridization.
   a. Significance of control in mRNA *in situ* hybridization. Interpretation of hybridization products as evidence of the presence of an identified segment of nucleic acid usually requires various controls. Specificity of the probes may be confirmed through the use of other modes of hybridization such as Northern blotting but a short length of the oligoprobes makes it difficult to perform Northern blotting with this probe. Therefore, the following controls for *in situ* hybridization should be prepared for appropriate interpretation of the findings ***(18)***.
   b. Positive controls of mRNA *in situ* hybridization. The first one is positive-control tissue, e.g., proliferative endometrium in the cases of mRNA *in situ* hybridization of ERα. This tissue should be simultaneously run in each assay, and positive hybridization reactions confirm the reaction of oligoprobes and various reagents employed in mRNA *in situ* hybridization. The second one is positive-control oligoprobe. This probe should hybridize with a target present in all tissues such as poly-T probe and should be labeled in the same manner and diluted in the same concentrations as the oligoprobes employed. Positive mRNA *in situ* hybridization reactions in the tissue sections demonstrated preservation of mRNA in the cells and availability to oligoprobes employed.
   c. Negative controls of mRNA *in situ* hybridization. Negative control probe is also required for appropriate interpretation of mRNA *in situ* hybridization. Oligonucleotide probes with sense orientation is usually employed for this purpose. This probe should also be labeled in the same manner and diluted in the same concentrations as the oligoprobes employed. Negative results in the same tissue specimens demonstrated specificity of hybridization and detection.

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V

Cancer Research and Drug Discovery

22

Solid Tumor Cancer Markers and Applications to Steroid Hormone Research

Marcia V. Fournier, Katherine J. Martin, and Arthur B. Pardee

1. Introduction

Steroid hormones bind to their specific nuclear receptor protein, which are bound to their DNA receptor motifs and become activated to turn on transcription of other genes ***(1)***. As an important example, estrogen binds to estrogen receptors (ERs) and activates the progesterone receptor gene ***(2)***. Similarly, testosterone binds to its receptor and activates the prostate specific antigen gene, *PSA*, and others ***(3)***.

In a given cell, not all of the approx 30,000 genes produce their corresponding mRNAs and proteins, under a given set of conditions at any time. Activities of only a few of these genes are altered by a change of condition, such as by addition or removal of a steroid hormone.

The genes that become activated can be identified by application of several novel techniques. Described here are procedures based upon the differential display technique ***(4)*** for discovery of gene expressions that change in response to hormone activation. The authors illustrate applications of this method with discovery of effects of estrogen on gene expressions in normal vs breast cancer cells. Estrogen activates growth and gene expressions of ER+ tumor cells that overexpress ERs, compared to normal breast or ER– tumor cells that do not express this receptor. Solid tissues are one set of targets to be investigated. And, with this technique, ER and other tumor-related gene expressions can be found in blood samples from cancer patients, compared to blood of healthy individuals.

A practical application of gene expression studies is to help decide upon appropriate therapy. Antihormones, such as tamoxifen, can be applied against ER+ breast tumors, but they are not useful against ER– ones. Discoveries of

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novelly expressed genes could permit earlier detection, early effects of experimental drugs and clinical treatments, and lead to novel therapies and to understanding of basic mechanisms of disease.

2. Materials

2.1. Basic Protocol for Differential Display

1. Sensiscript reverse transcriptase (RT) (QIAGEN, Valencia CA).
2. RNA Image® kit (GenHunter, Nashville, TN).
3. Differential display (DD) primers: Three anchor primers (T11N) and eight 13–20-bp arbitrary primers.
4. Glycogen 10 mg/mL stock solution in dH_2O.
5. TE buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* ethylerediamine tetraacetic acid (EDTA).
6. AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ).
7. *Taq* Polymerase chain reaction (PCR) buffer 10X solution: 100 m*M* Tris-HCL, pH 8.4, 500 m*M* KCl, 15 m*M* $MgCl_2$, and 0.01% gelatin.
8. Deoxyribonucleoside trilphosphates (NTPs): stock solutions of 2.5 m*M* and 250 μ*M* in dH_2O.
9. α-(^{33}Pdeoxycytidine triphosphate [dCTP]), 3000 Ci/mol (DuPont NEN, Boston MA).
10. Mineral oil.
11. Isopropanol.
12. 70% Ethanol.
13. GenomyxLR DNA sequencer (Genomyx, Foster City CA).
14. 3 *M* NaOAc.
15. Circum Vent Sequencing Kit (New England BioLabs, Beverly, MA).
16. [γ-^{32}P]adenosine triphosphate (ATP) 5' (DuPont NEN).
17. Synthetic 20–40-bp oligonucleotides complementary to appropriate region of genes of interest, which can be end-labeled and used to probe Northern blots or PCR amplify from total cellular RNA.
18. Assess to databases.

2.2. Two-Step Method for Identifying Tumor Markers in Biopsy Samples

1. Agarose.
2. PCR purification column.
3. 96-Well PCR dish.
4. 10 *M* NaOH.
5. 0.5 *M* EDTA.
6. Positively charged nylon membranes.
7. Multiprint 96-pin replicator with 16 offset positions (V&P Scientific, San Diego, CA).

8. PhosphorImaging screen.
9. Ethanol.
10. ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA).
11. Formamide.
12. 0.5 μg/μL oligo$(dT)_{12–18}$ (Gibco-BRL, Life Technologies).
13. ^{32}P αdCTP (DuPont NEN).
14. SuperScript II (Gibco-BRL, Life Technologies).
15. G-50 columns (Boehringer Mannheim).
16. 1 *M* Tris-HCl.
17. 2 *N* HCl.
18. Wash solution: 5 mL 20X standard sodium citrate + 10 mL 20% sodium dodecyl sulfate (SDS) in 2 L total volume.
19. Software ImageQuant (Molecular Dynamics, Sunnyvale, CA).

2.3. Strategy to Study Potential Tumor Markers in Peripheral Blood Samples

1. 10 m*M* Tris-HCl, pH 7.6, 5 m*M* $MgCl_2$, 10 m*M* NaCl solution.
2. Trizol (Gibco-BRL, Life Technologies).
3. Red cell lysis solution (Ambion).
4. Ficoll Plaque Plus (Pharmacia Biotech).

3. Methods

3.1. Basic Protocol for DD

3.1.1. DD Protocol

1. Perform RT reaction. A clear, readable cDNA pattern was produced using Omniscript or Sensiscript RT (Qiagen) with as little as 16 ng total RNA, Sensiscript RT was 50-fold more sensitive than was SuperScript II RT (Gibco-BRL) ***(5)***.
2. DD may be performed using the RNA Image kit (GenHunter). Additional arbitrary primers, not included in the GenHunter kit, were recently described ***(6,7)***. It is suggested to perform all PCRs in duplicate. Make a 20-μL final volume PCR reaction for each primer combination. Set up the reaction mix on ice containing 0.2 μL 5 U/μL AmpliTaq DNA polymerase (Perkin-Elmer), 0.2 μL α-[^{33}P]dCTP, 2.0 μL RT mix from **step 1**, 2.0 μL 2 μ*M* anchor primer, 2.0 μL 2 μ*M* arbitrary primer, 1.6 μL 25 μ*M* dNTP, 2.0 μL 10X PCR buffer, 10.0 μL ddH_2O. Mix well by pipeting up and down. Add mineral oil if needed. PCR at 94°C for 30 s, 40°C for 2 min, 72°C for 30 s. For 40 cycles, followed by 72°C for 5 min. Keep reaction 4°C.
3. Electrophorese duplicate PCR products in parallel on extended-format denaturing 6% polyacrylamide gels (Genomyx). Excise bands of interest from the gel.
4. For purification of the cDNA from polyacrylamide fragments, add 100 μL TE buffer, pH 8.0, to the fragments. Incubate 10 min at room temperature. Boil

20 min. Spin 2 min at 16,000*g*. Remove supernatant to a fresh tube. Add 10 µL 3 *M* NaOAc, 3 µL 10 mg/mL glycogen and precipitate in 2 vol of ethanol.

3.1.2. PCR and Direct Sequencing

1. Perform PCR reactions of isolated cDNA fragments with 2.5 U/µL AmpliTaq DNA polymerase (Perkin-Elmer), 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 1.5 m*M* $MgCl_2$ and 250 µ*M* each of dNTP and 50 n*M* of each primer (same pair used for DD) in 50 µL reaction mix. The PCR reaction may be programmed as follows: 95°C 36 s, 53°C 36 s, 72°C 90 s for 30 cycles; elongation at 72°C for 5 min, and refrigeration at 4°C. A second round of the PCR reaction may be performed, when necessary. The PCR products of cDNA fragments should mostly be single bands.
2. Determine the nucleotide sequences of cDNA fragments using the Circum Vent Sequencing Kit (New England BioLabs) with [γ-^{32}P] rATP 5' end-labeled primers, as described in the supplier's instructions. The DNA template should be purified from an agarose gel (QIAquick). Run the samples on a 6% polyacrylamide gel (Genomyx Corporation, Foster City, CA) at 60°C, 3000 V, 125 W. Automated sequencing is also indicating when available (*see* **Note 1**).
3. Sequences may now be queried against National Center for Biotechnology Information databases, using the Basic Local Alignment Search Tool (BLAST) ***(8)***.
4. Following database verification, design a single gene-specific 20-mer primer, and use in combination with the appropriate DD anchor primer to PCR-amplify a homogeneous probe for Northern blotting, or reverse Northern. This strategy of generating homogenous probes for confirmation of DD results showed that the method does not generate a significant number of false positives ***(9)***.

3.2. Two-Step Method for Identifying Tumor Markers in Biopsy Samples

The authors have developed a two-step approach to identifying tumor markers in biopsied tissue samples. In particular, the markers found by this approach accurately determined the ER status of breast tumor ***(10)***. It uses, as a first step, an approach to discovery novel gene, DD, in the authors' case, to compare normal vs tumor cells and to identify differentially expressed genes. These differentially expressed genes include many that are important in the process of tumorigenesis. In addition, the cell types of interest express all of these genes; hence, they are candidate marker genes. The approach uses as a second step an array-based method to efficiently screen hundreds of genes at once for their expression patterns in clinical tumor samples. Data from the arrays are analyzed by statistical comparisons of individual genes or by cluster analysis to analyze groups of genes.

3.2.1. Membrane-Based Hybridization Arrays

This procedure is composed of three parts: preparation of replicate membranes, preparation of ^{32}P-labeled first-strand cDNA, and membrane hybridization.

3.2.1.1. Preparation of Replicate Membranes

To produce replicate membrane arrays with tags for up to 384 different genes, PCR products or whole plasmids are spotted using a handheld 96-pin spotting devise. Twenty membranes are a convenient number to make at once.

1. PCR-amplify gene tags to produce DNA concentrations at least 0.1 μg/μL. Check an aliquot on an agarose gel. Purify using a commercially available PCR purification column.
2. Add 15 μL amplified DNA (or plasmids at ~1 μg/μL) to each well of a 96-well PCR dish. To each well, add 0.6 μL 10 *M* NaOH, and 0.3 μL 0.5 *M* EDTA. Denature tags 5 min at 95°C in thermocycler with heated lid, then chill on ice. To make more than 20 replicate membranes, larger volumes of DNA will be needed.
3. Wet with ddH_2O and blot-dry positively charged nylon membranes cut to the size of a 96-well dish. Apply denatured DNA tags to the membrane using a multiprint 96-pin replicator with 16 offset positions (V&P Scientific). Apply multiple spots of each gene tag (e.g., quadruplicates). It is crucial that the pin-spotting device is thoroughly cleaned regularly during the arraying procedure. To clean: Dip pins in dilute detergent, apply brush, rinse well in ddH_2O, dip in ethanol, and flame to dry. Clean pins at least between every three membranes.
4. Crosslink gene tags to membrane by UV irradiation (1200 μJoules). Dip in ddH_2O for 5 s. Store at 4°C in sealed plastic bags.

3.2.1.2. Preparation of ^{32}P-Labeled First-Strand cDNA

1. Thaw total cellular RNA on ice. The authors typically run experiments with six different RNAs at once, which is a convenient number to work with, and six membranes will fit together on a single, large PhosphorImaging screen.
2. Prehybridize membranes in 5.0 mL ExpressHyb (Clontech) solution at 68°C, or in 5 mL formamide buffer at 40°C, for at least 1 h using roller bottles.
3. To a PCR tube, add 2 μL 0.5 μg/μL oligo$(dT)_{12–18}$ (Gibco-BRL), and 5.0 μg RNA. Add diethyl pyrocarbonate (DEPC)-treated ddH_2O to 12 μL total volume. Denature by heating at 70°C for 10 min in thermocycler, then place on ice. Centrifuge briefly.
4. To the denatured RNA, add 2.5 μL 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl, 2.5 μL 25 m*M* $MgCl_2$, 2.5 μL 0.1 *M* dithiothreitol, 1.0 μL 20 m*M* deoxyguanosine triphosphate/ATP/TTP, 1.0 μL 120 μ*M* dCTP, 5.0 μL ^{32}P (dCTP fresh), and mix well. Incubate at 42°C for 5 min in thermocycler.
5. Add 1.0 μL SuperScript II (Gibco-BRL). Incubate at 42°C for 50 min.
6. Meanwhile, prepare prepacked G-50 columns (Boehringer Mannheim) by centrifuging at 3000 rpm for 3 min as described by manufacturer. Also prepare a fresh solution of 3 *M* NaOH.
7. Following 50 min incubation, stop the RT reaction by adding 3.0 μL 3 *M* NaOH. Incubate at 68°C for 30 min, then add 10.0 μL 1 *M* Tris, 3.0 μL 2 *N* HCl, and 4.0 μL ddH_2O, and mix well. To determine percent incorporation of ^{32}P, remove 1.0 μL to a tube labeled "before" and estimate the total volume of the remaining reaction.

8. Place a collection vial below the G-50 column, and add the RT reaction to the column. Centrifuge column at 3000 rpm for 4 min. Remove 1.0 μL after centrifugation to a separate tube labeled "after." Estimate the total volume and note any significant changes in volume. Count radioactivity emitted from the "before" and "after" tubes and use these values and volume values to calculate percent incorporation. Percent incorporation should be 3–30%.

3.2.1.3. Hybridization of Replicate Membranes

1. Denature the ^{32}P-labeled first strand cDNA at 95–100°C for 2–5 min, then quickly chill on ice. Add labeled cDNA directly to the buffer in the hybridization tube and swirl gently to mix.
2. Incubate in rotating hybridization oven at 68°C, if using the Clontech ExpressHyb buffer, or at 40°C, if using a formamide-based buffer. Hybridize for 12–18 h. The authors have found that the use of a formamide-based hybridization buffer will produce markedly lower backgrounds, and allow more repeated uses of membrane. Formamide-based hybridization buffer (makes 2 L): To autoclaved 4-L Erlenmeyer flask with stir bar add 134.4 g Na tetraphosphate (×7 H_2O), 500 mL DEPC-treated ddH_2O. Adjust pH to 7.2 with phosphoric acid (about 6 mL). Add 100 mL 5 *M* NaCl, 140 g SDS (ultra-pure), and 4 mL 0.5 *M* EDTA pH 8.0. Incubate at 65°C overnight to dissolve. Bring temperature down to 42°C, and add 200 mL 50% dextran sulfate, 1 L 100% formamide (Fluka), and 20 mL 10 mg/mL autoclaved salmon sperm DNA boiled before adding. Aliquot into 50-mL tubes and store at 4°C.
3. Wash membranes by first rinsing them briefly, one at a time, in 500 mL wash solution (5 mL 20X standard sodium citrate + 10 mL 20% SDS in 2 L total volume). Perform three subsequent 500-mL washes, 10 min/wash, at 50°C, with the remaining 1500 mL of wash solution. Agitate while washing and wash, at most, three membranes per 500 mL wash solution. Blot to semi-dry membranes and wrap in plastic wrap. Place on PhosphorImaging screen for 24-48 h. An example of a pair of hybridized membranes is shown in **Fig. 1**.
4. Strip isotope by pouring 90°C water over membranes and leaving them in it (at room temperature) for 30 min. Membranes can be reused up to four times.

3.2.2. Analysis of Hybridization Array Data

1. Quantify signal intensities of hybridized spots with the phosphorimager by drawing equal size ellipses around all spots using software (ImageQuant) provided (Molecular Dynamics). Subtract median background. Enter these data to an Excel table.
2. Signals that are too low to measure accurately (e.g., less than fivefold above background) should be indicated as such (e.g., "BKG"). Calculate average signals from replicate (e.g., quadruplicate) measurable spots only if at least three of four spots were measurable. Disregard sets (genes) with standard deviations exceeding a cut-off value (e.g., 150% of the mean). Normalize signal intensities

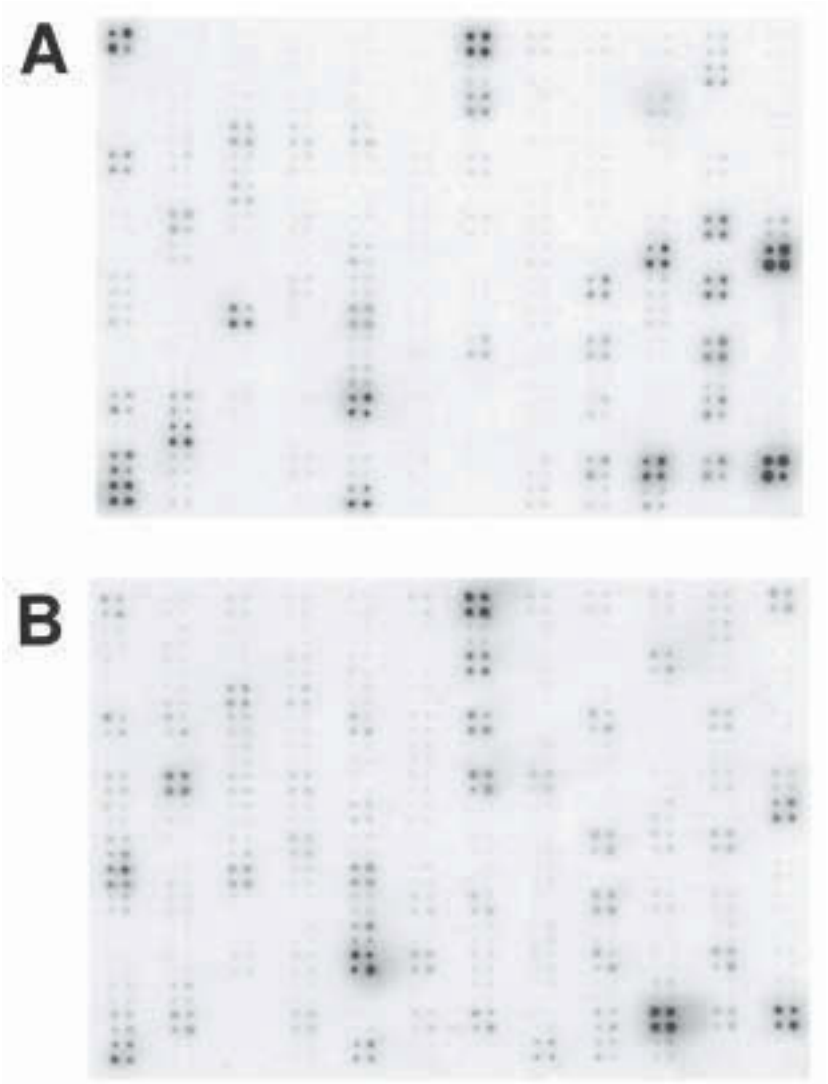

Fig. 1. High-density hybridization arrays. Two replicate membranes were hybridized in parallel with ^{32}P-labeled cDNA prepared from breast tumor cell lines. All gene tags are represented in quadruplicate. **(A)** ER+ cell line, ZR-75-1. **(B)** Estrogen receptor negative cell line, MDA-MB-157.

for each membrane to median signal of that membrane. To calculate average for RNAs run multiple times, determine geometric means of all non-BKG membrane-normalized values.

3. For ^{32}P hybridization arrays, it is important that signals that are too low to accurately measure are properly entered into succeeding calculations. A single-median BKG value should be determined for an entire set of membranes being compared. This value should be substituted for all BKG values.
4. Normalize signals for each individual gene to the geometric mean of the expression level of that gene across the set of membranes being compared. Omit genes with consistently low signals across an entire set of comparison membranes from the analysis.
5. Analyze patterns of expression of individual genes, using appropriate statistical tests.
6. Hierarchical cluster analysis can be performed using publicly available software written by M. Eisen, Stanford University (.edu/clustering). Data sets must be logarithmically transformed.

3.3. Strategy for Study of Potential Tumor Markers in Peripheral Blood Samples

Metastasis is the main basis of cancer deaths. The study of the gene expression pattern of circulating solid tumor cells opens a view to better understand tumor progression, and may be applied to identification of new molecular markers for tumor dissemination and metastasis ***(11,12)***. Tumor cells are released to the peripheral blood circulation each day. The study of circulating solid tumor cells in blood samples from cancer patients provides an in vivo picture of gene expression associated with tumor dissemination and invasiveness, but not every tumor cell is able to invade and disseminate in blood vessels. For this reason, the population of tumor cell circulating in blood is supposed to be less heterogeneous then at the primary and metastatic sites. Analysis of genetic expression on this set of cells potentially enhances the sensitivity to pick up metastasis-related genes. In addition, tumor dissemination is an essential step for metastasis formation, so it is likely to happen in more aggressive or advanced stages of malignancy. Although the methodology focuses on analysis of circulating solid tumor cells, it could be readily applied to any steroid-responsive gene identification in blood samples (*see* **Note 2**).

3.3.1. Preparation of RNA from Whole Blood **(1)**

1. To prepare total RNA from whole blood (WB), obtain 3–5 mL venous blood with a standard venipuncture technique using anticoagulant.
2. Centrifuge WB at 1800*g* for 40 min in a clinical centrifuge. Collect the cells present in the buffy coat and wash them with 3–5 mL buffer containing 10 m*M* Tris-HCl, pH 7.6, 5 m*M* $MgCl_2$, and 10 m*M* NaCl. Centrifuge at 1800*g* for 1 min and repeat this step three times. At the end of the first wash, remove one-half of the supernatant and bring it to the original volume with fresh buffer. After the last wash, remove the maximum supernatant possible.
3. Add 1 mL Trizol (Gibco-BRL) to the pellet containing nucleated cells (follow manufacturer's instructions) (*see* **Notes 3** and **4**).

3.3.2. Preparation of RNA from WB **(2)**

1. Place 40 mL of commercially available red cell lysis solution (Ambion) in a 50-mL conical tube. Add up to 10 mL EDTA WB to the tube and vortex thoroughly. Store tube on ice for 5–10 min, vortexing briefly two to three times during incubation.
2. Centrifuge tube for 3 min at 400*g* in a large centrifuge (r = 170 mm). Remove the supernatant by aspiration and discard.
3. Add 1 mL red cell lysis solution to pellet. Resuspend the pellet of leukocytes and residual red blood cells by pipeting. Transfer to microcentrifuge tube and centrifuge for 30 s at 16,000*g*.
4, Remove all supernatant, including the more opaque red layer that may be present directly over the leukocyte pellet. Leave no more than about 30 μL of residual fluid, but do not disturb pellet. Sometimes the pellet will include some reddish-brown material.
5. Use commercially available minipreparation methods (e.g., Trizol reagent) to isolate RNA. Use reagent as described by the manufacturer (*see* **Notes 3** and **4**).

3.3.3. Comparing Blood Samples by DD

1. The gene-expression pattern variation between individual samples must not distort the ability of DD to distinguish genes related to metastasis. Compare 3–5 normal control blood samples by DD, and choose the primer combinations (anchor x arbitrary) that generate a cDNA pattern with low variability between the controls. Once primer pairs are determined, search for specific tumor markers in blood samples, comparing test samples with controls. Limit sample number for comparison by DD. Compare 3–5 controls against 3–5 test samples: A greater number of samples may make analysis difficult when looking for potential tumor markers in blood samples. After choosing potential markers, extend analysis on them to as many samples as necessary.
2. To choose potential markers, one should look for cDNA fragments completely absent in control samples and present in at least two/thirds of test samples by DD.
3. Confirm DD results (*see* **Subheading 2.1.**).
4. Perform quantitative method for validation of potential markers. The authors suggest using Northern blot, semiquantitative RT-PCR, or quantitative RT-PCR. Because high sensitivity is required for detection of solid tumor markers in blood samples, real-time PCR methodology is strongly indicated. In the authors' experience one can detect approx one solid tumor cell in a million white blood cells by semiquantitative RT-PCR ***(12)***. However, other publications found limiting detection by PCR assays as sensitive as a single cell expressing a tumor marker among 10–100 million lymphocytes ***(13)***.

3.3.4. Cell Spiking and DD

Cell-spiking experiments are used to test the detection limit of DD of tumor cells in blood. **Figure 2** shows a section of a DD gel demonstrating a sensitive

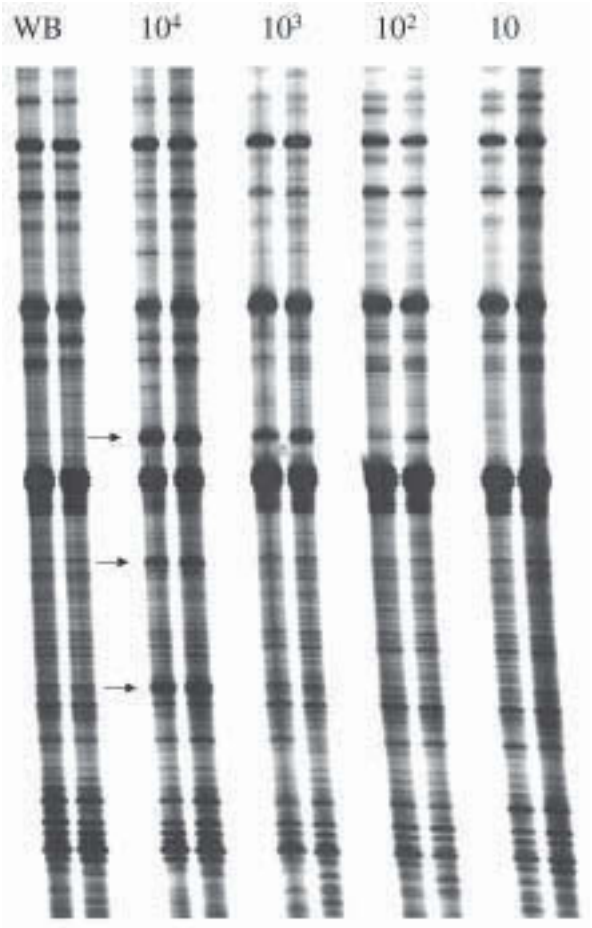

Fig. 2. Section of a DD gel demonstrates the sensitivity to detect differential expression of tumor cell genes in blood samples. Arrows show three differential expressed cDNA fragments in samples containing HeLa cells. RNA was isolated after mixing 10^4, 10^3, 10^2 or 10 HeLa cells with 3 mL WB.

detection of tumor cell genes in blood samples containing sequential dilutions of HeLa cells.

1. Add known numbers of a cell line (nonlymphoid origin) to 3 mL of WB prior to buffy coat separation. Perform RNA extraction as indicated above for blood samples.
2. Perform DD and confirm results by RT-PCR with specific primers.

4. Notes

1. Both primers, arbitrary and anchor, may be applied to sequencing analysis.
2. The same idea presented in this chapter for studying solid tumor markers in blood samples by DD may be applied for other gene discovery assays, e.g., using cDNA

arrays, serial analysis of gene expression (SAGE) analysis, and subtractive hybridization. Recently, Wan et al. ***(14)*** compared three methods for cloning differentially expressed mRNAs, including DD and subtractive hybridization. They concluded that DD is the method of choice because it identifies mRNAs independent of prevalence, uses small amounts of RNA, identifies increases and decreases of mRNA steady-state levels simultaneously, and has rapid output.

3. Treating RNA samples with DNase I is essential. Measure the RNA spectrophotometrically by making OD_{260} and OD_{280} readings of 1:250 dilutions (2 in 500 µL DEPC-ddH_2O). Ratio should be >1.6. $OD_{260} \times 10 = \mu g/\mu L$. It is important to check the quality and the quantity of RNA by running 1–2 µg on an agarose gel.
4. Do not hold blood for more than 12 h (at 4°C) prior to RNA isolation. White cells can be isolated, then frozen at –70°C indefinitely. 5 mLWB yields ~3×10^7 white cells, ~6–10 µg RNA.

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23

Assessing Modulation of Estrogenic Activity of Environmental and Pharmaceutical Compounds Using MCF-7 Focus Assay

Kathleen F. Arcaro and John F. Gierthy

1. Introduction

The MCF-7 cell line was isolated from a pleural metastasis of a human breast adenocarcinoma, and, when grown on plastic substrates, typically forms a continuous cell monolayer at confluence ***(1)***. MCF-7 cell cultures respond to 17β-estradiol (E_2) by increases in the expression of a number of genes ***(2,3)*** and localized focal postconfluent cell proliferation, which results in development of multicellular, three dimensional nodules termed "foci" ***(4)***. Thus, focus development in MCF-7 cells may represent the basic characteristics of an estrogenic response, i.e., induction of concerted gene expression, resulting in tissue restructuring through enhanced postconfluent cell proliferation. Since foci are easily counted, the development E_2-induced foci and their inhibition are useful as a relevant human-tissue-based assay for the assessment of estrogenic and antiestrogenic activity of environmental and pharmaceutical compounds ***(5–7)***. Here the authors give the protocol for measuring focus formation in response to estrogen-modulating agents. In addition, protocols are presented to determine whether the modulation of foci by a particular agent is a result of estrogen-receptor (ER)-dependent activity or changes in the level of E_2 through alteration of E_2 catabolism. **Table 1** provides an overview of the three protocols.

2. Materials

1. Equipment: Standard tissue-culture equipment including a laminar flow hood, inverted microscope, and an incubator set at 37°C with 5% CO_2, 95% air are needed. Either an automated colony counter or a plate reader is needed to quantify the foci (*see* **Note 1**). Access to a scintillation counter is needed for both the

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Table 1
Overview of Protocols for Assays

	Seed (cells/mL/well)	Refeed (after seeding)	Incubation with $[^3H]E_2$	Endpoint	Measurement
MCF-7 Focus assay	1×10^5	24 h and every 3–4 d for a total of 4 refeeds	None	Development of foci Inhibition of focus development	Increase or decrease in focal retention of rhodamine B stain
Whole-cell ER-binding assay	5×10^5	24 h	For 3–4 h; at the same time as test agent or a specified time after test agent; at either 4 or 37°C	Displacement of $[^3H]E_2$ from ER by test agent	Decrease of $[^3H]E_2$ in cells, with increase in test agent
Radiometric analysis of catabolism of $[^3H]E_2$	5×10^5	24 h	Between 3 and 24 h; after incubation with test agent for a series of time points; at 37°C	Increase in tritiated catabolism of $[^3H]E_2$, resulting in production of tritiated H_2O	Increase in tritiated H_2O in media, with increase in test agent

whole-cell competitive ER-binding assay and the radiometric analysis of E_2 catabolism.

2. MCF-7 cell line: We routinely use an MCF-7 strain obtained from Dr. Alberto C. Baldi, Institute of Experimental Biology and Medicine, Buenos Aires, Argentina. However, MCF-7 cells obtained from the American Type Tissue Culture Association have also successfully used. Both these lines are known to be heterogeneous, and enhanced performance, if required, can be achieved by generating single cell clones ***(4)***.
3. Culture media: Media supplies are available from Sigma (St. Louis, MO), Gibco-BRL Products (Grand Rapids, MI), and Hyclone (Logan, UT). Standard tissue culture medium is made as follows: Supplement 1 L Dulbecco's modified Eagles medium (high glucose, no phenol red) with the following: 5% bovine calf serum (*see* **Note 2**), 10 ng/mL insulin, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 m*M* nonessential amino acids, and 2 m*M* L-glutamine. Filter medium through a 500 mL vol, 0.2-µm pore-size plastic Nalgene filter unit (Nalgene, Rochester, NY), and store in sterile glass bottles.
4. Plastic tissue culture supplies: When possible, plasticware (15- and 50-mL centrifuge tubes for storing refeeding media, flasks, and plates) should be polypropylene as opposed to polystyrene. The authors and others ***(8)*** have encountered problems with leaching of estrogenic compounds from polystyrene plastic. At present, the authors have not found a supplier of polypropylene flasks or tissue culture plates.
5. Reagents/buffers/solutions:
   a. 0.05% Trypsin (Gibco) with 0.025% EDTA in phosphate buffered saline (PBS) is needed for detaching cells from culture flasks.
   b. Formalin (10% formaldehyde in PBS and 1% rhodamine B in PBS are needed for fixing and staining cultures for the focus assay).
   c. Absolute ethyl alcohol is needed to solubilize bound E_2.
   d. Charcoal slurry, needed for the whole-cell ER-binding assay, is prepared in 50-mL tissue culture tubes as follows: Mix charcoal and dH_2O (1:2 v:v) and centrifuge (about 1000*g*). Remove supernatant, and wash again to remove the fines. Resuspend the washed charcoal in H_2O (1:2, v:v).
   e. [2,4,6,7,16,17-^{3}H]E_2 (specific activity, 140–150 Ci/mmol) and [2,4,6,7-^{3}H]E_2 (specific activity, 70 Ci/mmol), available from NEN (Boston, MA), are used in the whole-cell competitive binding and E_2 catabolism assays, respectively (*see* **Note 3**). Biodegradable scintillation cocktail is available from Econo-Safe (Mount Prospect, IL).

3. Methods

Standard cell culture techniques are used to maintain T-75 flasks of MCF-7 cell cultures. Newly confluent flasks are used for seeding experiments.

3.1. MCF-7 Focus Assay

The basic focus assay takes 14 d from seeding to fixing and obtaining results. The assay can be completed in a shorter time (7 d or less) by seeding the wells at a higher cell density. However, this should be done only if it is known that the test concentrations of the agent(s) of interest are not cytotoxic. The authors routinely run the focus assay in 24-well plates (other plates can be used), and a typical experiment will include 6–12 plates. Every experiment includes a plate with an E_2 concentration–response curve, and every plate includes a column (4 wells) with a vehicle control as shown in **Fig. 1**. Below is given the protocol for the standard 14-d focus assay run in 24-well plates.

1. Prepare single-cell suspension: Remove medium from a 75 cm^2 flask and add 4 mL of a 37°C trypsin solution, rotate flask back and forth to cover all areas with the solution, remove 2 mL of the solution and discard. Tighten the cap and return the flask to the incubator for approx 10 min. Gently shake the flask to check the attachment of the cells; if cells do not detach in a curtain, return to the incubator. If cells are detached from the substrate, add 10 mL tissue culture medium to the flask and prepare a single-cell suspension by repeatedly drawing the cells into and out of a 10-mL tissue culture pipet (*see* **Note 4**).
2. Seed plates: Count the number of cells in 100 μL of the cell suspension using an automated cell counter or a hemacytometer. Add the appropriate amount of the cell suspension to tissue culture medium to obtain a concentration of 1×10^5 cells/mL needed to seed the desired number of plates (*see* **Note 5**). Add 1 mL of the cell suspension to each well of the 24-well plates. Place the seeded plates in the incubator and do not disturb for 24 h.
3. Prepare refeeding media: The standard-response curve includes five concentrations of the test agent and a vehicle control with four replicates each (*see* **Figs. 1** and **2**). Since each well in a single condition is refed four times with 2 mL each time, a total of 32 mL is needed for each condition. A concentration series of the test compound is prepared in either ethanol or dimethyl sulfoxide and diluted 1:1000 into the tissue culture medium; thus, all test media contain 0.1% of the vehicle. For tests of antiestrogenicity, prepare media with 0.1 n*M* E_2 (the minimal concentration of E_2 necessary to induce a maximum response) and the test agent. Store refeeding media in the refrigerator and warm to 37°C before refeeding cells.
4. Refeed cells: Cultures in each well are fed the experimental medium 24 h after seeding, then three additional times (every third day, for a total of four refeeds). Aspirate the medium 24 h after seeding in the well and quickly replace it with 2 mL test medium. Aspirate and refeed one plate at a time to minimize drying of the culture and cell death. Return the plates to the incubator. During the next 2 d, visually examine the cells with an inverted microscope to check for cytotoxic effects indicated by changes in morphology, such as pycnosis, lysis, or detachment, and estimate the percent of the well surface covered by cells (percent

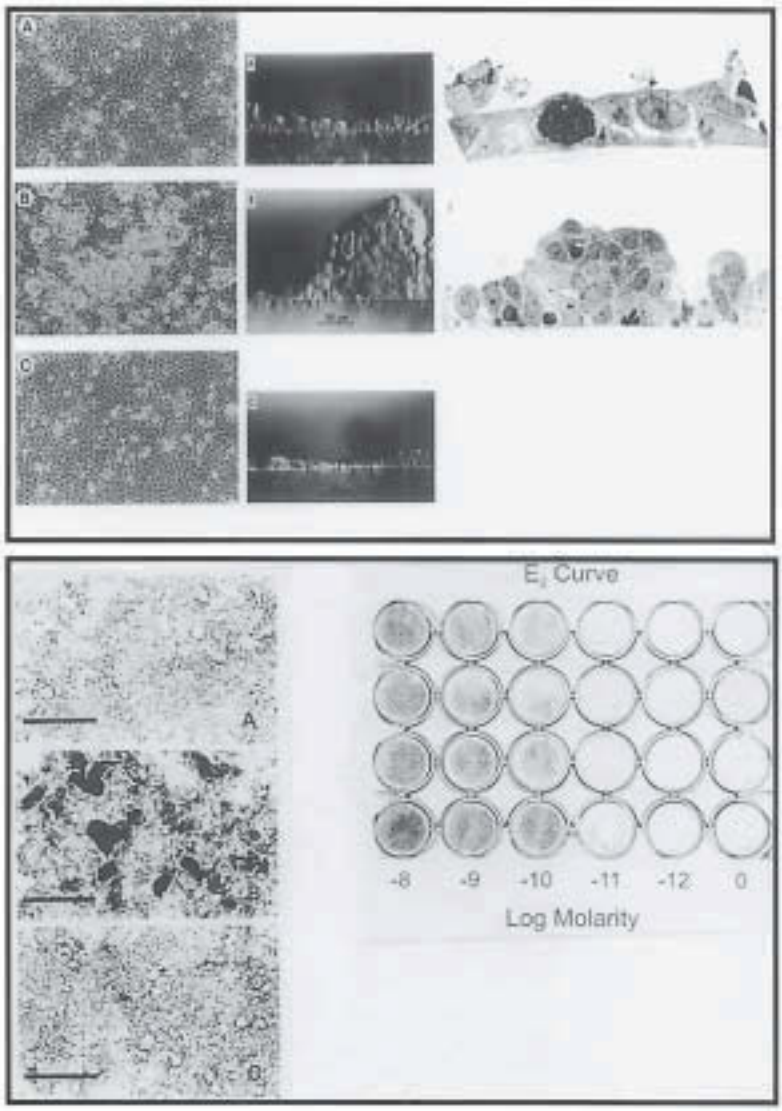

Fig. 1. Induction of focus development by E_2 in MCF-7 cultures. *Upper panel*: (left) phase contrast 100X; (center) side view Nomarski optics; (right) side view transmission electron microscopy. *Lower panel*: (left) light microscopy of fixed rhodamine B stained culture; (right) typical E_2 dose-response focus assay in the 24-well format. The red rhodamine B stain appears as black in the lower panel because of stain retention by the multicellular foci. Lettered panels are: **(A)** vehicle control (0.1% DMSO); **(B)** 1 n*M* E_2 treatment; **(C)** 1 n*M* E_2 with 1 n*M* of the antiestrogen 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD).

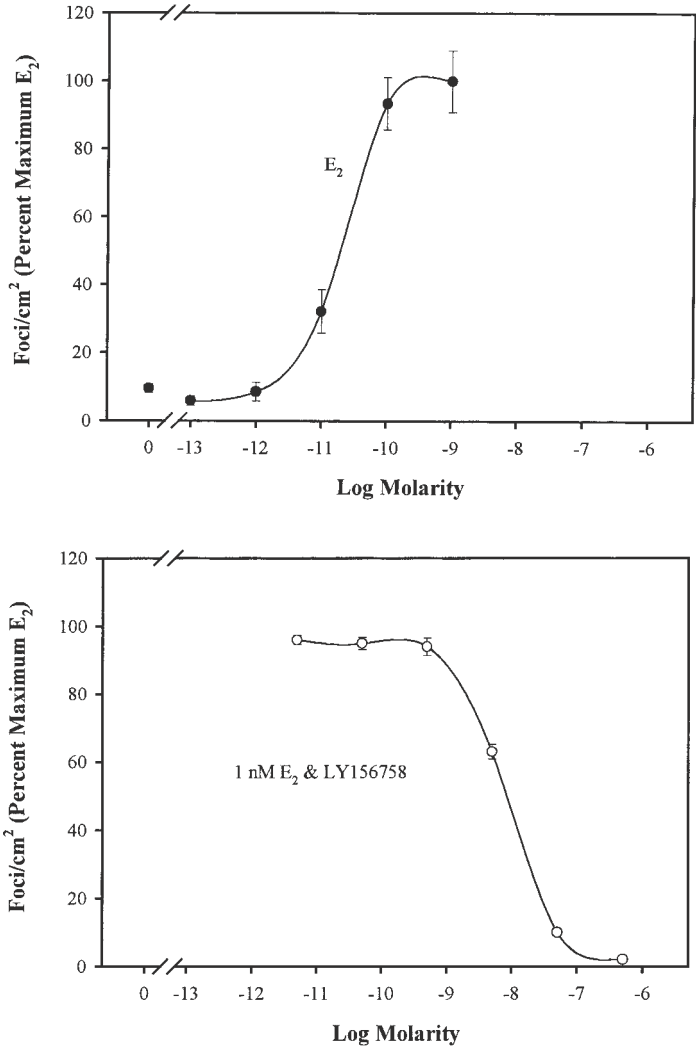

Fig. 2. *Upper panel:* Induction of focus development by E_2 in MCF-7 cultures. *Lower panel*: Inhibition of E_2-induced focus development by the antiestrogen LY156758.

confluent). Date of confluence can be used as an indication of cytostatic effects. If the test agent does not cause toxicity or delay growth, then cells in all wells should reach confluence on the same day as the control culture. By checking for confluence, one can discriminate between agents that are antiestrogenic (inhibit the formation of E_2-dependent foci) and those that are cytotoxic or cytostatic (kill cells or delay preconfluent growth). Cells should reach confluence 3–4 d after seeding.

5. Fix, stain, and count foci: After 14 d, remove media from all wells and add 1 mL formalin to each well for 1 h to fix the cells. Remove formalin; put plates in fume hood to dry. When dry, add 1 mL of 1% rhodamine B to each well and leave for 1 h. Collect the stain by carefully inverting the plate over a plastic container slightly larger than the plate. (The stain can be returned to the stock bottle and reused.) At this point, the stain in cells in all wells will appear uniformly intense.

   Destain the cells by rinsing in tap water. Destaining highlights the multicellular foci against the monolayer background because the dye in the monolayer background rinses away more quickly than the dye in the multicellular foci. Fill a plastic tub with room-temperature water and maintain under running tap. Dip each plate vertically into the tub gently filling each well, empty, and repeat three times. Set plates with filled wells on bench. After 10 min, repeat the rinsing procedure. Continue rinsing until the control plate, the E_2 concentration-response curve, shows a clear E_2–curve (*see* **Fig. 1**, lower panel). At this point, stop rinsing all plates. Invert and shake the plates over the sink to remove liquid and place on bench to dry. Drying can be hastened by using a hair dryer. When dry, the foci are counted using an automated colony counter or other device (*see* **Note 1**).
6. Interpret results: The authors routinely normalize the data to the maximum E_2 response (**Fig. 2**). This allows comparisons among different experiments. Control-level background is not subtracted; thus, the data convey the signal-to-noise ratio, which should be approx 10 for a maximal E_2 response.

3.2. Whole-Cell Competitive ER-Binding Assay

This assay can be conducted at both 4°C and 37°C. Conducting the assay at 4°C inhibits the metabolism of the test compound and ensures that any activity results from the parent compound as opposed to a metabolite of the parent compound. Conducting the assay at 37°C allows for potential metabolism of the parent compound, possibly from an inactive compound (one that does not bind the ER) to an active compound (one that binds the ER). Below, the protocol is given for examining the binding of parent compounds at 4°C (*see* **Note 6** for conducting the assay at 37°C).

1. Seed plates: The whole-cell ER binding assay is performed 24 h after seeding; therefore, seeding is done at a higher density, to yield confluent wells within 24 h. Prepare a single-cell suspension; count 100 µL cell suspension; add the necessary amount of cell suspension to culture medium to produce the needed concentration of 5×10^5 cells/mL/well. Add 1 mL cells to each well (*see* **Note 7**). Place in incubator for 24 h.
2. Prepare experimental media: Experimental media contains 1 n*M* [2,4,6,7,16,17-^{3}H]E_2 and varying concentrations of either unlabeled E_2 or the test agent. Include a 200-fold excess E_2 control and a vehicle control.
3. Refeed cells: Remove plates from incubator, aspirate medium from wells, and replace with 1 mL 4°C experimental media containing test compound and 1 n*M*

[^{3}H]E_2. Wrap the edge of the plate with parafilm to prevent evaporation and place in 4°C refrigerator for 4 h or overnight, depending on the rate at which the test agent enters the cell and the nucleus.

4. Extract bound [^{3}H]E_2: Remove plates from refrigerator, and warm to room temperature. Carefully aspirate media from wells, and rinse twice with 1.5 mL room-temperature PBS. Aspirate all PBS and add 300 µL ethanol to each well (use an accurate repeating pipetor) to solubilize the bound [^{3}H]E_2. Let stand for 15 min and mix. Remove 200 µL ethanol extract from each well and place in scintillation vial. Add scintillation cocktail and determine radioactivity.
5. Interpret results: Determine the amount of bound [^{3}H]E_2 in the presence or absence of the test compound by subtracting the nonspecific binding. The nonspecific binding equals the amount of bound [^{3}H]E_2 in the presence of 200-fold excess unlabeled E_2. Express data as the ratio of bound [^{3}H]E_2 in the presence of a competitor to the bound [^{3}H]E_2 in the presence of the vehicle control × 100. Because the wells were seeded at a high cell density and left to attach overnight, there should be little test agent-dependent cell growth and the number of cells/well should be close to 5×10^5, with little variability among wells. Cell number assessment of replicate plates will confirm this.

3.3. Radiometric Assay for [^{3}H]E_2 Catabolism

This assay is a simple and relatively inexpensive method for determining whether test agents increase or decrease the catabolism of E_2 and is particularly useful in determining whether inhibition of foci observed in the focus assay is caused by decreased levels of E_2. The radiometric assay measures the amount of tritium released into the medium as tritiated H_2O from any and all of the labeled positions (*see* **Note 3**).

1. Seed plates: Prepare cells and seed plates 5×10^5 cells/mL/well.
2. Prepare refeeding media: Media is needed for one refeed only. Refeeding media contain the test compound in standard culture medium.
3. Refeed cells: After 24 h, exchange culture medium for test media and return plates to the incubator. After the desired incubation with test media (6, 12, 24, 60, or 72 h), remove plates from the incubator. Using a 10-mL sterile pipet, carefully remove the 2 mL medium from the four replicate wells of each treatment group and pool. Place 4 mL/well of this pooled conditioned medium in a sterile tube with [2,4,6,7-^{3}H] E_2 for a final concentration of 1 n*M* [2,4,6,7-^{3}H]E_2. Return 1 mL of the radioactive, pooled medium to each of the four replicate wells. Because it is important to quickly remove and replace the medium from the wells, the needed [2,4,6,7-^{3}H]E_2 must be aliquoted into tubes ahead of time. Return the plates to the incubator for 24 h.
4. Measure displaced [^{3}H]E_2: Using a repeating pipetor, add 200 µL of charcoal slurry to 1.5-mL centrifuge tubes (one tube for each well). Remove the plates from the incubator. Remove 200 µL media from each well of the 24-well plates, and add to the charcoal slurry in centrifuge tubes. Vigorously mix for 30 min on

a batch vortexer. Pellet charcoal by centrifugation (2500*g* for 15 min at 2–8°C). Place 100 µL supernatant in a scintillation vial, add scintillation cocktail, and determine radioactivity.

5. Determine cell count: Cell count can be determined by adding trypsin solution to each well, preparing a single cell suspension, and counting with an automated cell counter or a hemacytometer. This method, however, is time-consuming. Alternatively, cell density can be estimated based on a colorimetric assay ***(9)*** as follows. Remove all media from wells; add 100 µL 10% trichloracetic acid to each well to fix; rinse with H_2O, and air-dry; add 1 mL 0.4% (w/v) sulforhodamine B dissolved in 1% acetic acid to each well for 30 min; remove dye; rinse four times quickly with H_2O to remove unbound dye; extract bound dye with 100 µL of 10 m*M* unbuffered Tris-base for 1 h. Determine optical density of extracted dye with a spectrophotometer at 564 nm.
6. Interpret results: The authors generally present data from the radiometric analysis of E_2 catabolism as counts/min/10^6 cells ***(10)***. It is important to conduct a concentration response curve with multiple incubation times of the test compound, because many of the compounds that induce the enzymes that catalyze the catabolism of E_2 are also substrates for the induced enzymes and may compete with and even inhibit the catabolism of E_2 at one concentration, while inducing the catabolism at another concentration ***(10)***.

4. Notes

1. The foci can be counted directly, using an automated colony counter, such as the AccuCount 1000, available from BioLogics (Gainesville, VA). The foci can also be determined indirectly by resuspending the dye accumulated in the foci in 1 mL H_2O (overnight incubation), and measuring the density of the dye spectrophotometrically or fluorometrically. In the authors' experience, the greater dynamic range of the fluorometer (absorbance: 550 nm; emission: 580 nm) yields better results. For 96-well plate readers, transfer 0.1 mL from each of the 24 wells.
2. Media can be made with either whole or stripped bovine calf serum. The low level of endogenous E_2 in calf serum as opposed to fetal bovine serum provides an approximation of a low E_2 adult environment and does not require stripping ***(4)***. The authors routinely run the assay with whole bovine calf serum.
3. Until recently, the authors used [2-^{3}H]E_2, from NEN Life Sciences in the radiometric E_2 catabolism assay because of specific interest in degradation of E_2 at the 2 position only. However, this product is no longer available and another supplier has not been found. Either [2,4,6,7-^{3}H]E_2 or [2,4,6,7,16,17-^{3}H]E_2 can be used in the radiometric assay.
4. Obtaining a single-cell suspension is important; seeding aggregates may result in their attachment and misinterpretation as foci. Obtaining a single-cell suspension by repeatedly drawing the cells into and out of a 10-mL pipet may be difficult. Check the flask under an inverted microscope to assure that you have a single-cell suspension. If you do not have a single-cell suspension, use a sterile 18-gage

hypodermic needle on a 10-mL syringe, and repeatedly draw the cells gently through the needle until a single-cell suspension is obtained.

5. A guideline for determining the number of confluent flasks needed to seed the desired number of plates is as follows. One confluent T-75 flask contains roughly 10^7 cells; 24×10^5 cells are needed to seed a single 24-well plate at 1×10^5 cells/mL/well; therefore, one confluent flask seeds roughly four plates for the MCF-7 focus assay. If the cell count revealed that you have 8×10^5 cells/mL in 10 mL, and two plates are to be seeded, add 6.25 mL cell suspension to 43.75 mL culture medium for 50 mL 1×10^5 cells/mL. All wells should receive the same number of cells. Use an accurate, large-volume, repeater pipet to add 1 mL cell suspension to each well. Make certain the cells remain evenly dispersed in the seeding medium, by continued mixing of the cell suspension until seeding of the plates is completed. It is critical that the newly seeded plates are placed in the incubator and left undisturbed before the cells settle to the well surface. Any movement after the cells have settled and before they have attached to the substrate will result in uneven cell density. The authors advise seeding no more than three plates at a time before placing them in the incubator.
6. Conducting the whole-cell competitive ER-binding assay at 37°C allows for metabolism of the test agent and may detect metabolites of the parent compounds that bind the ER. Incubate the cell cultures with the test compound (without [^{3}H]E_2) for the desired length of time at 37°C. Remove plates from incubator and cool to 4°C. Remove media from replicate wells, quickly mix with [^{3}H]E_2 for a final concentration of 1 n*M* [^{3}H]E_2, and return the media to the wells. Wrap edges of plate with parafilm and return to 4°C refrigerator for 3 h, continue with **Subheading 3.2., step 4**.
7. Often, the biggest problem with these assays and other assays examining long-term growth in cell culture is the greater evaporation that occurs in the outside wells which can result in altered growth of the replicate cultures in the outer wells, possibly because of a concentration of nutrients in the medium, and may cause problems interpreting data. The uneven growth can be even more pronounced in 96-well plates (*see* **ref. *11*** for a discussion of parabolic growth patterns in 96-well plates and possible causes for this phenomenon). The uneven evaporation and growth occurs even though the plates are maintained in 90% humidity in the incubator. The authors have used a number of methods for overcoming this problem when it arises. The focus assay can be conducted in 48-well plates, leaving the entire outer ring of wells filled with water. The inner wells, 6 columns of 4 replicates each, result in a functional 24-well plate. Alternatively, or in addition, to further help maintain humidity, the plates can be placed inside a plastic container (Rubbermaid, 3.78 L) fitted with a plastic 10-cm high grid on the bottom. After the container is filled to a depth of 10 cm with H_2O, the entire container is placed in the incubator. Appropriate holes cut in the lid of the container facilitate gas exchange while limiting the evaporation. This arrangement is particularly useful when several people are using the incubator throughout the day.

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Combinatorial Chemistry in Steroid Receptor Drug Discovery

John A. Flygare, Daniel P. Sutherlin, and S. David Brown

1. Introduction

Combinatorial chemistry has significantly increased the speed of small molecule lead discovery and optimization for multiple targets in the pharmaceutical industry. Large libraries of bioactive compounds, synthesized either as mixtures or discreet units, can now be made using solid- or solution-phase techniques ***(1–4)***. The design of the libraries can be biased toward a specific lead structure, enabling the rapid dissemination of information about the compound of interest ***(5,6)***. This chapter describes the methods developed for the application of combinatorial chemistry to the solid-phase synthesis of steroid receptor ligands.

Combinatorial chemistry has been successfully applied to several members of the nuclear receptor superfamily of transcription factors. Peroxisome proliferator-activated receptor (PPAR) ligands have been synthesized using solid-phase techniques. In this procedure, chemistry was developed to synthesize a lead compound for this orphan receptor on the solid-phase. In one example, the library of compounds synthesized using this chemistry led to the discovery of a high-affinity subtype selective ligand for PPARδ ***(7,8)***. Using a different lead structure, solid-phase techniques were used to improve the potency of PPARγ ligands ***(9)***. Within the steroid hormone receptor family, solid-phase combinatorial chemistry has been used to synthesize estrogen receptor ligands ***(10–12)***. Work has also recently been directed at making estrogen receptor ligands more amenable to combinatorial synthesis ***(13)***. Tamoxifen (1) is a high affinity ligand for the estrogen receptor, containing an aromatic subunit similar to the A-ring phenol in naturally occurring estrogens

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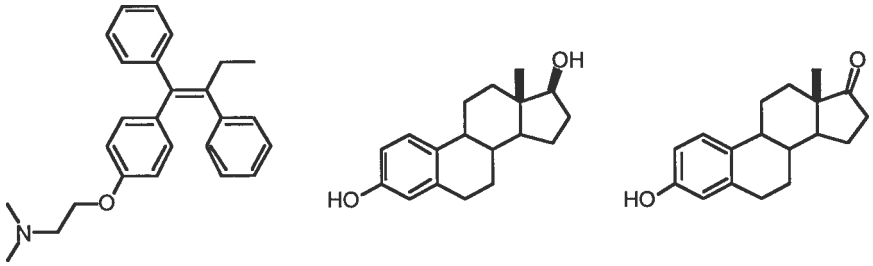

Fig. 1. Tamoxifen (1) and the naturally occurring estrogens 17β-estradiol (2) and estrone (3).

(**Fig. 1**). This chapter details the synthesis of tamoxifen and related compounds on the solid-phase.

2. Materials

All chemical reagents are available from Aldrich (Milwaukee, WI) and are used as received, except dimethoxyethane, which was purchased from Fisher Scientific and used directly.

2.1. General Materials

1. Tetrahydrofuran (THF).
2. Dimethylformamide (DMF).
3. Dichloromethane (DCM).
4. Methanol.
5. Ethyl acetate (EtAc).
6. Nitrogen gas.
7. High vacuum pump.
8. $MgSO_4$.
9. Na_2SO_4.

2.2. Linker Synthesis

1. Diiodobenzene.
2. *n*-butyl lithium in THF.
3. Allyldimethylsilyl chloride.
4. 9-Borabicyclo [3,3,1] nonane (9-BBN).
5. 3 *M* KOH.
6. 30% H_2O_2 in H_2O.
7. Pyridinium dichromate.
8. pH 10.0 NaOH.
9. Concentrated HCl.

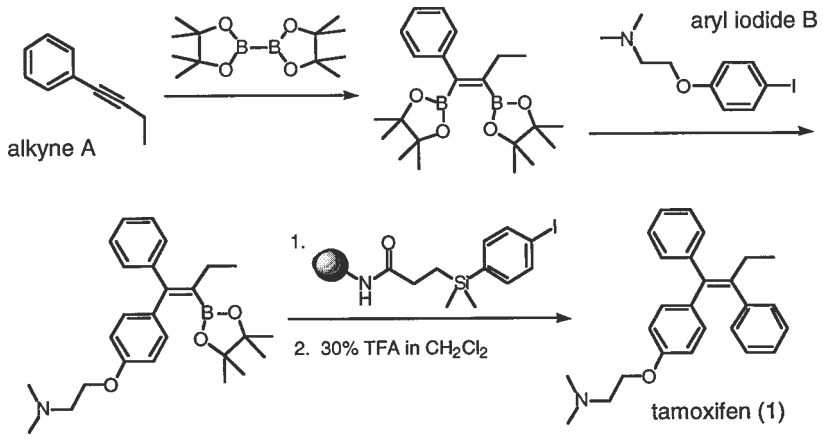

Fig. 2. The resin-based strategy to synthesize tamoxifen (1).

2.3. Resin Synthesis

1. Argogel amine resin available from Argonaut Technologies (San Carlos, CA).
2. 1-Hydroxybenzotriazole.
3. *N*,*N*-Diisopropylcarbodiimide.

2.4. Synthesis of Bis(boryl)alkenes

1. Various alkynes.
2. *Bis*(pinacolato)diboron.
3. Tetrakis(triphenylphosphine)platinum.
4. Hexane.

2.5. Synthesis of Triarylethylenes on Solid Support

1. Various aryl halides.
2. 3,5-Dimethoxyphenol.
3. Pd(dppf)Cl_2.
4. 6 *M* KOH.
5. Dimethoxyethane.
6. 30% Trifluoroacetic acid (TFA) in CH_2Cl_2.

3. Methods

A schematic for the following transformations is presented in **Fig. 2**. The majority of combinatorial chemistry examples in the literature carry out many of the synthetic transformations on the solid support. This work demonstrates the concept of resin capture, in which much of the chemistry is performed off the resin with a final step that captures only a single desired chemical element

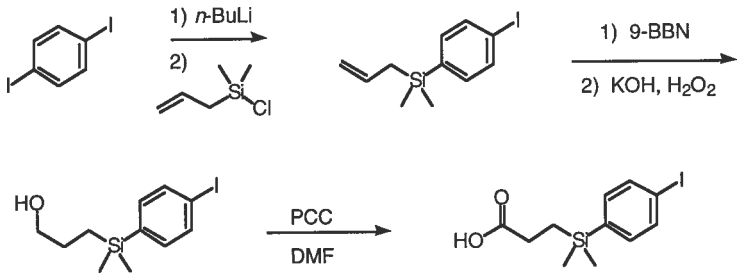

Fig. 3. Synthesis of the traceless linker.

onto the solid support. This helps to eliminate side products and results in the isolation of pure material upon cleavage from the resin.

The synthesis utilizes two diversity units in the procedure, an alkyne (A) and an aryl iodide (B). Many different alkynes and aryl iodides can be used in this sequence, providing a library of potential estrogen receptor ligands. Therefore, the method is presented using a generic alkyne and aryl iodide to allow users to insert their desired compounds.

3.1. Synthesis of Traceless Linker

This linker represents one of many available to the combinatorial chemist. Many are commercially available. The key feature of this linker is the hydrogen-for-silicon replacement that occurs during the cleavage process. As a result, no trace of the linker is present in the cleaved material. The synthesis is outlined in **Fig. 3**.

1. Cool 5 g diiodobenzene (1 Eq) in 75 mL THF to –40°C under a N_2 atmosphere.
2. Add *n*-butyllithium (10.2 mL 1.5 *M* solution in hexanes, 15.3 mmol, 1.01 Eq) followed by allyldimethylsilyl chloride (2.25 mL, 1.02 Eq) each over a 5-min period.
3. Warm the mixture to 0°C and add 9-BBN (4.10 g, 1.1 Eq) in 40 mL of THF slowly and allow to stir for 6 h at room temperature.
4. Cool the reaction to 0°C and add 6 mL 3 *M* KOH and 6 mL of 30% H_2O_2.
5. Remove the cold bath and let the reaction stir overnight.
6. Extract the organic layer with brine and dry it with $MgSO_4$, filter, and evaporate.
7. Dissolve the residue in 250 mL of DMF and add 25 g pyridinium dichromate (4.4 Eq) before stirring overnight.
8. Pour the reaction into 250 mL of water and extract five times with ether.
9. Wash the combined organic layers five times with aqueous NaOH (pH = 10.0).
10. Combine the aqueous layers and acidify with concentrated HCl and extract five times with ethyl acetate.
11. Dry the combined organic layers over Na_2SO_4 and evaporate to give 2.11 g 3-(1-[4-iodophenyl]-1,1-dimethylsilyl)propanoic acid as a white solid. ^1H nuclear

magnetic resonance (500 MHZ, $CDCl_3$): δ 0.28 (m, 6H), 1.07 (m, 2H), 2.30 (s, 2H), 7.21 (m, 2H), 7.70 (m, 2H).

3.2. Preparation of Iodophenyl Resin

1. Combine 4.0 g Argogel amine resin (0.43 mmol/g loading, 1 Eq), the solid from 3.1 (2.0 g, 5.98 mmol, 3.5 Eq), and 1-hydroxybenzotriazole (0.890 g, 6.59 mmol, 3.8 Eq) in 20 mL of dimethylformamide (*see* **Note 2**).
2. Add *N,N*-diisopropylcarbodiimide (1.05 mL, 6.71 mmol, 3.9 Eq) and stir the suspension slowly for 5 h.
3. Wash the resin with DMF (3 × 20 mL), DCM (3 × 20 mL), EtAc (3 × 20 mL), methanol (3 × 20 mL), and DCM (3 × 20 mL).
4. Dry the resin under vacuum.

3.3. Synthesis of Bis*(boryl)*alkenes

1. In 25 mL DMF combine an alkyne (3 mmol) with *bis*(pinacolato)diboron (0.770 g, 3.03 mmol) and tetrakis(triphenylphosphine)platinum (0.115 g, 0.092 mmol), and heat to 80°C for 18 h under a N_2 atmosphere.
2. Evaporate the DMF and dissolve the residue in hexane.
3. Wash the hexane with water and brine.
4. Dry with Na_2SO_4 and evaporate to dryness to give the *bis*(boryl)alkene.

3.4. Synthesis of Triarylethylenes on Solid Support

1. Flush a test tube with N_2 and cap with a rubber septum.
2. Combine a *bis*(boryl)alkene (0.38 mmol) with an aryl halide (0.57 mmol) in 1 mL of dimethoxyethane.
3. Add a solution consisting of 3,5-dimethoxyphenol (0.293 g, 1.90 mmol), Pd(dppf)Cl_2 (0.016 g, 0.02 mmol), 0.32 mL 6 *M* KOH, and 0.5 mL dimethoxyethane and let sit for 18 h.
4. Add 100 mg of resin from **Subheading 3.2.** and an additional 0.63 mL 6 *M* KOH.
5. After an additional 18 h, filter the resin, and wash it with water (3 × 10 mL), methanol (3 × 10 mL), DCM (3 × 10 mL), ETAc (3 × 10 mL), and DCM (3 × 10 mL) (*see* **Note 3**).
6. Treat the resin with 2 mL 30% TFA in DCM, filter and evaporate to give the product as a mixture of E and Z isomers (*see* **Note 4**).

4. Notes

1. Although the resin prepared in **Subheadings 3.1.** and **3.2.** is used in this instance, other resin attachment methods could also be used, as in **ref. *11***.
2. The amide coupling used to prepare the resin can be monitored by the standard ninhydrin test.
3. The resin must be exhaustively washed with various solvents prior to initiating the next step. The final wash should be done with a solvent that properly swells the resin, such as DCM. If the resin is not swelled in this last wash, problems may occur in the next step.

4. In the case of tamoxifen and other amine-containing products, the TFA salt will be isolated after **step 6**. The salt can be dissolved in 1 mL of DCM and passed through a small plug of basic alumina, to generate the free amine.

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Identification of Nuclear Receptor Interacting Proteins Using Yeast Two-Hybrid Technology

Applications to Drug Discovery

Sunil Nagpal, Corine R. Ghosn, and Roshantha A. S. Chandraratna

1. Introduction

The yeast two-hybrid system is a powerful tool for the isolation and characterization of nuclear receptor interacting proteins such as coactivator and corepressor proteins. Generally, coactivators associate with the nuclear receptors in an agonist-dependent manner and this specific protein–protein interaction forms the basis for ligand-mediated transcriptional activation of the target hormone-responsive genes. The association of corepressors with unliganded nuclear receptors allows the target genes to be in a repressed state in the absence of agonists. However, depending on the stoichiometry of nuclear receptor coactivator and corepressor proteins in a given cell, some degree of receptor/coactivator interaction can occur, resulting in a basal level of gene transcription. The ability of antagonists to maintain this basal equilibrium interaction or to increase receptor–corepressor interaction will determine whether they function as neutral antagonists or inverse agonists.

Recently, a number of nuclear receptor cofactors have been identified both by biochemical and yeast two-hybrid expression cloning strategies. These cofactors include *RIP140* ***(1–3)***, *RIP160* ***(1,3)***, *CBP/p300* ***(4)***, *Sug1/Trip1* ***(5,6)***, *Src-1/N-CoA1* ***(7)***, *TIF2/GRIP1* ***(8,9)***, *ACTR* ***(10)***, *N-CoR* ***(11)***, and *SMRT* ***(12)***. Of these, *N-CoR* and *SMRT* are corepressors ***(11,12)*** and *CBP/p300*, *SRC-1*, *GRIP1*, and *ACTR* are coactivators ***(4,7–10)***.

The ability of coactivators to potentiate the transcriptional activity of nuclear receptors suggested that they may change chromatin structure when recruited

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by liganded receptor to the promoter regions of responsive genes. Since acetylation of core histones may result in the destabilization of nucleosomes resulting in depletion of the nucleosomal core at the promoter regions of transcriptionally active genes, a number of laboratories looked into the possibility of coactivators functioning directly as histone acetyltransferases (HATs). *CBP* ***(13)***, *p300* ***(14)***, *SRC-1* ***(15)***, and *ACTR* ***(10)*** were found to act as HATs in vitro. Thus, ligand-dependent recruitment of HAT activity to a promoter can contribute to the transcription inducing activities of agonists. Expression cloning techniques in yeast also resulted in the cloning of corepressors, *N-CoR* ***(11)*** and *SMRT* ***(12)***. These proteins were found to interact with Sin3 and histone deacetylase. Therefore, the interaction of the receptor with *N-CoR*(*SMRT*)–*Sin3*–histone deacetylase complex can lead to remodeling of chromatin to a more compact form and hence repression of transcription. The recruitment of this repressor complex to a nuclear receptor by an inverse agonist is a plausible mechanism for the repression of basal transcriptional activity.

The authors' laboratory has identified high-mobility group *(HMG)-I* and *HMG-R* as new cofactors ***(16)***, which associate with retinoid acid receptor *(RAR)* in a ligand dependent manner, and the receptor-interacting domain of *RIP140* (RID2) which interacts with *RARs* in an agonist-dependent manner. We demonstrate that yeast two-hybrid assays involving *RAR*–RID2, *RAR*–*HMG-I*, and *RAR–HMG-R* interactions can be used for the identification of receptor and function-selective retinoids. Further, agonist-dependent, nuclear-receptor–*HMG* interaction may explain the destabilization and depletion of linker H1 histones, which must occur as a first step at the promoter regions of transcriptionally active genes. Mechanisms by which transcription factors affect the removal of H1 histones from the silent chromatin have not been addressed before. *RAR*–RID2 interaction appears to correlate with the ability of retinoids to inhibit the growth of breast cancer cells. These results are addressed in detail in this chapter.

2. Materials

All yeast strains and plasmids for the two-hybrid system were obtained from Clontech (Palo Alto, CA) as components of the MATCHMAKER Two Hybrid System.

2.1. Plasmids

Yeast shuttle vectors containing the GAL4 DNA-binding domain (DBD) (pAS2 and pGBT9) and the GAL4 activation domain (pACT2) were from Clontech.

2.2. Yeast Strains and Media

1. The *Saccharomyces cerevisiae* yeast stain Y190 (*MAT*α*, ura 3-52, his 3-200, ade 2-101, lys 2-801, trp 1-901, leu 2-3, 112, gal4*Δ*, gal80*Δ*, cyh 2, LYS2::GAL1*$_{UAS}$*–HIS3*$_{TATA}$*–HIS3, URA3::GAL1*$_{UAS}$*–GAL1*$_{TATA}$ *–lacZ*) (Clontech MATCHMAKER system II) was grown at 30°C in either YPD medium or synthetic defined drop-out (SD) yeast medium lacking the appropriate amino acids, i.e., tryptophan, leucine and histidine (Clontech). All the media contained glucose (2%) as a source of carbon. YPD agar medium or SD agar medium was also purchased (Clontech), and the plates prepared, as described by the manufacturer.
2. 3-Amino-1,2,4-triazole (3-AT) containing medium: Add required amount of 1 *M* 3-AT stock solution to medium below 55°C.
3. TTNPB: 5 m*M* stock solution of 4-(2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-2-propenyl) benzoic acid (TTNPB) prepared in acetone is added to media to give a final concentration of 1 μ*M*.

2.3. Buffers and Other Reagents

1. 10X *Taq* polymerase buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl.
2. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
3. 10X TAE: 400 m*M* Tris-HCl, 10 m*M* EDTA, pH 7.8.
4. 5X Dye: 15% Ficoll; 0.25% bromophenol blue, 5 m*M* K_2HPO_4.
5. 2X LB: 20 g tryptone; 10 g yeast extract; 10 g NaCl in 1000 mL water.
6. 2X LB glycerol: 175 mL 2X LB and 25 mL 100% glycerol.
7. TE lithium acetate (LiAc) buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0, and 100 m*M* LiAc.
8. Z buffer: 60 m*M* Magnesium (Mg) monohydrogen phosphate; 40 m*M* Na dihydrogen phosphate; 10 m*M* KCl; 1 m*M* Magnesium (Mg) sulphate, pH 7.0.
9. X-gal: Make a 20 mg/mL stock solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in *N,N*-dimethylformamide.
10. Z-buffer–X-Gal solution: 100 mL Z buffer; 270 μL β-mercaptoethanol; 1.67 mL X-gal stock solution.
11. ONPG solution: Make 4 mg/mL solution of *o*-nitrophenyl β-D-galactopyranoside (ONPG) in Z buffer.
12. Ampicillin: 50 mg/mL (1000X) stock solution in dH_2O.
13. Cycloheximide: 1 mg/mL (1000X) stock solution in dH_2O.

3. Methods

3.1. Preparation of the Bait Vectors

Bait vectors, pAS2 *RAR*αΔAB, pAS2*RAR*βΔAB, and pAS2*RAR*γΔAB contained amino acids 88–462, 81–448, and 90–454, respectively, of human *RAR*α, β, and γ, fused to the GAL4 DBD. These amino acid sequences spanned the C–F regions of the receptors. These regions were polymerase chain reaction (PCR)-

Table 1
Sequences of PCR Primers Used for Preparing Bait Vectors

Bait vector	PCR primers	Sequence
pAS2*RAR*αΔAB	A1	5'-AGGAATTCTGCTTTGTCTGTCAGGACAAGT-3'
	A2	5'-AGGGATCCTCACGGGGAGTGGGTGGCCGGG-3'
pAS2*RAR*βΔAB	B1	5'-AGCCCGGGGTGCTTCGTCTGCCAGGACAAATCAT-3'
	B2	5'-AGGGATCCTTATTGCAGGAGTGGTGACTGACTG-3'
pAS2*RAR*γΔAB	G1	5'-AGGAATTCTGCTTCGTGTGCAATGACAAGT-3'
	G2	5'-AGGGATCCTCAGGCTGGGGACTTCAGGCC-3'
pAS2*RAR*γ	g1	5'-ACCGCAGGGATCCTGGCCACCAATAAGGAGCGC-3'
	g2	5'-TAATGGATCCTGGTCAGGCTGGGGACTT-3'
pGBT*RXR*α	X1	5'-AGGAATTCATGGACACCAAACATTTCCTGCCG-3'
	X2	5'-AGCTGCAGCTAAGTCATTTGGTGCGGCGCCTC-3'

amplified using primer pairs A1 and A2 for pAS2*RAR*αΔAB, B1 and B2 for pAS2*RAR*βΔAB and G1 and G2 for pAS2*RAR*γΔAB. Full-length RARγ bait vector, pAS2*RAR*γ (amino acids 1–454) was prepared by PCR amplification of human *RAR*γ using oligos g1 and g2. Wild-type *RXR*α bait vector, pGBT*RXR*α (amino acids 1–462) was prepared by PCR amplification of human *RXR*α, using oligos X1 and X2. The nucleotide sequences of all the primer pairs are presented in **Table 1**.

1. Add 100 ng human *RAR* expression vector ***(17)*** to a thin-walled 500-μL microcentrifuge tube containing 100 ng appropriate primer pair (**Table 1**), 2 μL dNTP stock (dATP, dCTP, dGTP, and dTTP, 10 m*M* each), 5 μL 10X *Taq* polymerase buffer (Perkin-Elmer, Branchburg, NJ), 1 μL of *Taq* polymerase (Perkin-Elmer), 2 μL of 25 m*M* $MgCl_2$. Adjust the total volume to 50 μL with sterile H_2O. Mix the contents of the tube by flicking with a finger and spin at 1000*g* for 10 s.
2. Carry PCR amplification in Perkin-Elmer 9600 thermal cycler for 40 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. After the final cycle, incubate for 10 min at 72°C to ensure reaction completion.
3. Add 10 μL of 5X dye and load the mix on a 1% agarose gel in 1X TAE buffer. The gel contained ethidium bromide (2 μL of 10 mg/mL ethidium bromide/ 100 mL gel).
4. Purify the approx 1.0 kb PCR-amplified fragment by using QIAEX II DNA gel extraction kit (Qiagen, Valencia, CA).
5. Suspend the purified fragment in 20 μL TE.
6. To 7 μL purified fragment in a microcentrifuge tube, add 1 μL *Eco*RI, 1 μL of *Bam*HI, and 1 μL core buffer (Promega, Madison, WI) for restriction digestion. Mix the reaction.

7. Incubate at 37°C for 1 h.
8. Add 2 µL 10X dye, run on a 1% agarose gel, cut out the ethidium bromide-stained band, and purify the restriction-digested fragment using QIAEX II DNA gel extraction kit (Qiagen).
9. Digest pAS2 (bait vector) with the appropriate restriction enzymes.
   a. For cloning *RAR*αΔAB and *RAR*γΔAB fragments in the bait vector to 1 µg pAS2 (1 µL) in a microcentrifuge tube, add 1 µL *Eco*RI, 1 µL *Bam*HI, 1 µL core buffer, and 6 µL of sterile H_2O.
   b. For cloning *RAR*βΔAB in the bait vector, take 1 µg pAS2 (1 µL) in a microcentrifuge tube, add 1 µL *Sma*I, 1 µL *Bam*HI, 1 µL core buffer (Promega) and 6 µL of sterile H_2O.
10. Incubate at 37°C for 1 h.
11. Add 1 µL calf intestinal alkaline phosphatase (CIP), 5 µL 10X CIP buffer, and 34 µL TE.
12. Incubate at 37°C for 30 min followed by an incubation of 10 min at 65°C.
13. Add 50 µL TE, extract with phenol/chloroform, add carrier yeast tRNA (1 µg), ethanol-precipitate the DNA, centrifuge, wash the pellet with 70% ethanol, dry the pellet and suspend in 10 µL TE.
14. For ligation reaction, add 5 µL *RAR* fragment in a microcentrifuge tube. Add 1 µL restriction-digested pAS2, 2 µL 10X ligase buffer, 1 µL T4-DNA ligase and 11 µL TE.
15. Incubate the ligation reaction overnight at 15°C.
16. Transform 1 µL of ligation product in 40 µL DH12S electrocompetent *Escherichia coli* (Life Technologies) cells by electroporation using Bio-Rad *E. coli* pulser.
17. Plate *E. coli* on a LB agar plate with 50 µg/mL ampicillin and grow overnight at 37°C.
18. Identify positive clones with the *RAR* constructs, verify the clones by sequencing, and purify the bait constructs by double cesium chloride banding (*see* **Note 1**).

3.2. Transformation of Yeast

1. Inoculate Y190 in 1 mL YPD and grow overnight at 30°C.
2. Inoculate overnight grown Y190 into 50 mL YPD (*see* **Note 2**) and further grow for 3–5 h at 30°C to reach log phase (OD_{600} = approx 1).
3. Pellet the cells at 3000*g* for 5 min at room temperature (RT).
4. Wash the cells with 25 mL H_2O and centrifuge at 3000*g* for 5 min again.
5. Resuspend the cells in 0.4 mL TE lithium acetate buffer and aliquot 50 µL in microcentrifuge tubes.
6. For transformation, pellet the aliquoted cells, and resuspend in transformation mix containing 240 µL polyethylene glycol (50% w/v), 36 µL 1 *M* lithium acetate, 10 µg herring testis carrier DNA, and 1 µg pAS2*RAR*αΔAB, pAS2*RAR*βΔAB or pAS2*RAR*γΔAB.
7. Vortex the mixture for 1 min and incubate at 30°C for 30 min.

8. Heat-shock the cells at 42°C for 20 min.
9. Centrifuge at 5000*g* for 15 s, resuspend the pellet in 400 μL H_2O, and plate 200 μL on yeast–Trp-selective media plates.
10. Incubate the plates at 30°C for 3 d.

3.3. Amplification of Plasmid cDNA Library

A HaCaT keratinocyte library in pACT2 expressing GAL4-activation domain chimeras was purchased (Clontech). The keratinocyte library was amplified using the GeneTrapper protocol for semisolid amplification of plasmid cDNA libraries (*see* **Note 3**). The protocol is as follows:

1. Prepare 2 L 2X LB.
2. Add 500 mL 2X LB in autoclavable bottles and to each bottle add 1.5 g SeaPrep (FMC) agarose and a magnetic stir bar.
3. Autoclave for 30 min.
4. Cool the 2X LB agarose bottles to 37°C in a water bath.
5. Add 200 mg/mL ampicillin and keep on a stir plate for 2 min.
6. Inoculate each bottle with approx 300,000 primary cDNA transformants from the original cDNA library and mix by keeping on a stir plate for 2 min.
7. Place the bottles in an ice-water bath for 1 h.
8. Incubate the bottles at 30°C for 45 h.
9. Transfer the contents into centrifuge bottles and spin at 9800*g* for 20 min at RT. Make sure that the rotor and the centrifuge are at RT.
10. Pour off the supernate and use the pelleted cells to purify the plasmids by cesium chloride double-banding method.

3.4. Yeast Two-Hybrid Screening

1. Inoculate 3–4 colonies Y190 transformed with pAS2*RAR*γΔAB in 1 mL SD–Trp medium and grow overnight at 30°C (*see* **Note 4**).
2. Inoculate overnight grown Y190 into 50 mL SD–Trp medium and further grow for 3–5 h at 30°C to OD_{600} = approx 1.
3. Pellet the yeast cells at 3000*g* for 5 min at RT.
4. Resuspend in 25 mL H_2O and again centrifuge at 3000g for 5 min.
5. Resuspend the cells in 0.4 mL TE lithium acetate buffer and aliquot 50 μL in microcentrifuge tubes.
6. For transformation, pellet the aliquoted cells and resuspend in 240 μL peg (50% w/v), 36 μL 1 *M* lithium acetate, 50 μg herring testis carrier DNA, 8 μg keratinocyte library cDNA in pACT2-GAL4-activation domain plasmids and 60 μL sterile H_2O.
7. Vortex the mixture for 1 min and incubate at 30°C for 30 min.
8. Heat-shock the cells at 42°C for 20 min.
9. Centrifuge at 5000*g* for 15 s, resuspend the pellet in 1 mL SD-Leu-Trp-His medium.
10. Incubate for 30 min at 30°C.

11. Centrifuge for 15 s at 5000*g*.
12. Resuspend in 800 μL sterile H_2O and plate 100 μL on each SD-Leu-Trp-His selective media plate containing 25 m*M* 3-AT and 1 μ*M RAR*-specific retinoid TTNPB.
13. Incubate the plates at 30°C for 3–4 d.
14. Streak the colonies on SD-Leu-Trp-His plates (*see* **Note 5**) containing 25 m*M* 3-AT and 1 μ*M* TTNPB (*see* **Notes 6** and **7**).

3.5. β-Galactosidase Assays

Analyze the positive colonies (which grew on selective media plates in the previous steps) for their protein interactions by β-galactosidase activity. Measure β-galactosidase activity by two protocols, namely, colony-lift filter assay and an ONPG liquid assay. The former is a qualitative and the latter is a quantitative, measure of the protein–protein interactions.

3.5.1. Colony-Lift Filter Assay

1. Streak the yeast colonies, which grew on selective media on fresh SD-Leu-Trp-His plates containing 25 m*M* 3-AT and 1 μ*M* TTNPB.
2. Grow the yeast streaks at 30°C for 3 d.
3. Presoak 75-mm Whatman no. 5 filter paper with Z buffer–X-Gal solution.
4. Place the presoaked filter paper in a 100-mm plate.
5. Place a dry Whatman no. 5 filter paper over the surface of the yeast-streaked agar plate containing colonies to be analyzed for β-galactosidase activity (*see* **Note 8**).
6. Mark the orientation.
7. Lift the filter paper off the SD-Leu-Trp-His agar plate.
8. First place the filter paper with the yeast colonies facing up on the surface of liquid nitrogen, then submerge the paper in liquid nitrogen for 10 s.
9. Thaw the filter at RT to permeabilize the cells.
10. Place the filter, with colonies facing up, on the presoaked filter paper.
11. Incubate the filters at 30°C and check for the appearance of blue streaks, which indicate that the yeast cells contain the GAL4-activation domain-based interacting protein plasmid.

3.5.2. Liquid β-Galactosidase Assay

Subject the yeast colonies that are positive in filter lift assay to liquid β-galactosidase assay for quantitatively assessing the interactions between *RAR* and the interacting proteins. This step would also determine the ligand dependency of the protein–protein interactions.

1. Grow the yeast colonies in 5 mL SD-Leu-Trp medium overnight at 30°C with shaking.
2. Inoculate 2 mL overnight culture in 8 mL fresh YPD medium in the presence or absence of 1 μ*M* of the retinoid TTNPB.

3. Grow for 5 h (until mid-log phase) at 30°C and record OD_{600}. The OD_{600} of the 1-mL solution should be between 0.5 and 0.8.
4. Aliquot 1.0 mL culture into microcentrifuge tubes.
5. Centrifuge the tubes for 30 s at 17,000*g*.
6. Remove the supernate and resuspend each pellet in 1.5 mL Z buffer.
7. Centrifuge at 17,000*g* for 30 s.
8. Resuspend each pellet in 100 µL Z buffer.
9. The control tube should contain 100 µL Z buffer.
10. Dip the tubes in liquid nitrogen for 1 min.
11. Incubate at 37°C for 1 min.
12. Add 700 µL Z buffer containing β-mercaptoethanol (270 µL β-mercaptoethanol/100 mL Z buffer).
13. Add 160 µL ONPG solution to each tube (*see* **Note 9**).
14. Incubate at 30°C till the development of yellow color (*see* **Note 10**).
15. Record the time taken for the color to develop.
16. Stop the reaction by the addition of 400 µL 1 *M* Na_2CO_3.
17. Centrifuge the microcentrifuge tubes at 17,000*g* for 10 min to pellet the cell debris (*see* **Note 11**).
18. Transfer the supernatant to disposable cuvets and measure OD_{420}.
19. Calculate β-galactosidase units in which 1 β-galactosidase (β-Gal) unit is the amount of the enzyme required to hydrolyze 1 µmol ONPG to *o*-nitrophenol and D-galactose in 1 min.
20. Quantitate protein interactions in terms of β-Gal units using the following equation: β-Gal units = $1000 \times OD_{420}/(t \times OD_{600})$, where *t* is the time in min required for the development of the color.

3.6. Selecting Yeast Containing Fish (pACT2) Plasmid

1. Pick positive colonies, resuspend in 100 µL of sterile H_2O and spread on SD-Leu cycloheximide plates (*see* **Note 12**).
2. Incubate the plates at 30°C for 6–10 d.
3. Colonies that grow on these plates do not contain the GAL4 DBD plasmid (the bait plasmid).

3.7. Plasmid Isolation and Identification of Interacting Protein

1. Grow positive SD-Leu-Cyh (cycloheximide) plate yeast colonies in 5 mL SD-Leu medium for 20 h with shaking at 30°C.
2. Centrifuge at 1000*g* for 5 min at RT.
3. Pour off the supernatant and dissolve the pellet in the residual liquid.
4. Transfer to a fresh microcentrifuge tube.
5. Add 200 µL yeast lysis solution.
6. Add 200 µL phenol/choloroform/isoamyl alcohol (25:24:1) and 300 mg acid-washed glass beads.
7. Vortex for 2 min and centrifuge at 17,000*g* for 5 min at RT.

8. Transfer the supernatant to a microcentrifuge tube and add one-tenth vol of 3 *M* Na acetate (pH 5.2) and 2.5 vol ethanol.
9. Wash the pellet with 70% ethanol, dry under vacuum, and dissolve in 20 μL TE.
10. Use 2 μL to transform *E. coli*, plate on LB agar plate containing ampicillin, and grow the colonies overnight at 37°C.
11. Pick up the clones and isolate DNA for sequencing.
12. Sequence the plasmid cDNA and confirm the identity with the Basic Local Alignment Search Tool algorithm by running the cDNA sequence against the GenBank database.

3.8. Use of Yeast Two-Hybrid System in Drug Discovery

The yeast two-hybrid system can be used for the identification of the receptor-subtype and function-selective retinoids, and has the potential to replace the traditional eukaryotic cell transfection assays, which utilize *RAR* reporter and expression vector constructs, for the identification and systematic synthesis of receptor subtype selective retinoids. Further, this tool when taken together with the *RAR*-binding assays, has the capacity to identify function selective retinoids, which otherwise would be missed by transfection assays. These points are further elaborated by following data.

Using the yeast two-hybrid system with pAS2*RAR*γΔAB as the bait construct, the authors have isolated two types of proteins from a keratinocyte fish construct cDNA library, which interact with *RAR* in a ligand dependent manner. These proteins are *HMG-I* and *HMG-R*, *HMG* proteins ***(18,19)*** and *RIP140*, an estrogen receptor (*ER*)-interacting protein ***(2)***. The authors have identified two domains of *RIP140*, namely, RIDs 1 and 2, which interact with *RAR*γ in a ligand-dependent manner (Nagpal, Ghosn, and Chandraratna, unpublished observations). The authors have developed *RAR–HMG-I* and *RAR*–RID2 interaction assays for the identification of receptor subtype and function-selective retinoids. RID2 contained the C-terminal 906–1158 amino acids of *RIP140* and is a part of the region (amino acids 752–1158), which has previously been shown to interact with *ER* in an estradiol dependent manner ***(2)***.

3.8.1. RAR-HMG-I *Interaction*

Targeted recruitment of histone acetyltransferase (HAT) activity by *RARs*, other nuclear receptors and sequence-specific transcription factors, in general, has been proposed to be required for destabilization of the nucleosomal cores by acetylation of core histones (H2A, H2B, H3, and H4) ***(10,13–15)***. However, a number of studies in the past have indicated that the depletion of not only the core histones, but also of the linker histone H1 must be achieved at the promoter sequences of actively transcribing genes. The authors have recently

described a mechanism by which *RARs* and other transcription factors may displace H1 from their binding sites on chromatin ***(16)***, and have shown that *RARs* interact with *HMG-I* and a related novel protein, *HMG-R*, in a ligand-dependent manner. *HMG-I* is a nonhistone chromosomal protein, which binds to the A/T-rich sequences through a defined DNA binding domain called the A.T hook motif ***(18)***. *HMG-I* has three A.T hook motifs ***(18)*** in its protein sequence and *HMG-R* has two ***(16)***.

Histone H1 is a generalized repressor, that plays an important role in the compaction of chromatin into a transcriptionally silent fiber ***(20,21)***. *HMG-I* protein, by displacing histone H1 from its DNA binding sites, has been shown to derepress H1-mediated inhibition of transcription ***(22)***. Further, *HMG-I* is associated with transcriptionally active H1-depleted chromatin. *HMG-I* is also overexpressed in cancer cells, compared to normal cells ***(23–26)***. A number of groups have also demonstrated HMG-I interaction with various transcription factors, such as *nuclear factor-kB*, *activating transcription factor 2*, *Elf-1*, *Oct-2*, *Oct-6*/*Tst-1*, and *PU.1* ***(27–33)***. Taken together, these observations suggest that *RARs* and other nuclear receptors and transcription factors, may direct *HMG-I*/*HMG-R* to promoter regions of target genes. *RARs* and other nuclear receptors may perform this function in a ligand-dependent manner. Since displacement of H1 is a required primary event in order for transcription to take place, an *RAR–HMG-I*/R interaction assay would be a quantitative measure of a specific proximal event associated with gene transcription. This assay cannot only be used for the identification of *RAR*-subtype-selective agonists but also for the identification of retinoids with novel pharmacological profiles.

3.8.1.1. Ligand-Dependent Interaction of *HMG-I* with *RAR* and *RXR* Subtypes

Transform yeast cells with pAS2*RAR*αΔAB, pAS2*RAR*βΔAB, or pAS2*RAR*γΔAB and pACT2-*HMG-I* or pACT2-*HMG-R* expression vectors (*see* **Note 13**). Select the transformants on SD-Leu-Trp medium agar plates. As controls, transform yeast cells with the bait vector (pAS2*RAR*) or the fish vector (pACT2-*HMG*) alone. Subject yeast colonies to liquid β-Gal assay in the absence or presence of the TTNPB (a pan-*RAR* agonist) or an unknown ligand whose receptor specificity must be determined. As expected, yeast cells, expressing either the bait or the fish constructs alone, do not show any significant β-Gal activity in the absence or the presence of retinoid ligand. However, yeast cells transformed with both the bait and the fish vectors show a ligand-dependent increase in the expression of β-Gal reporter (**Fig. 1**). These results demonstrate that all three *RARs* interact with *HMG-R* and this assay can be used for the identification of receptor subtype selective agonists.

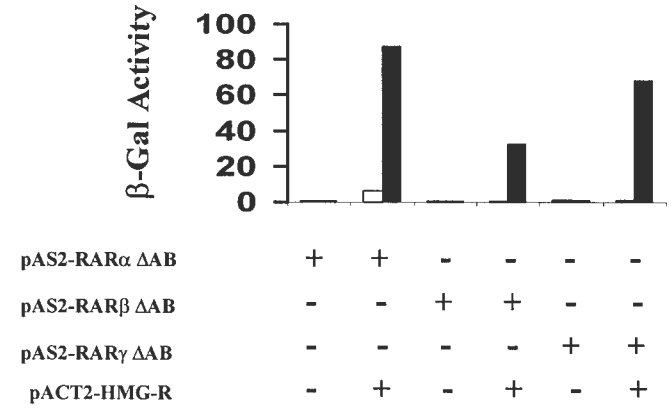

Fig. 1. *RARs* interact with *HMG-R* in a ligand-dependent manner. β-Galactosidase activities of yeast cells, cotransformed with *RAR* and *HMG-R* expression vectors in the absence (open bars) or presence (solid bars) of TTNPB (1 μ*M*) in yeast are shown.

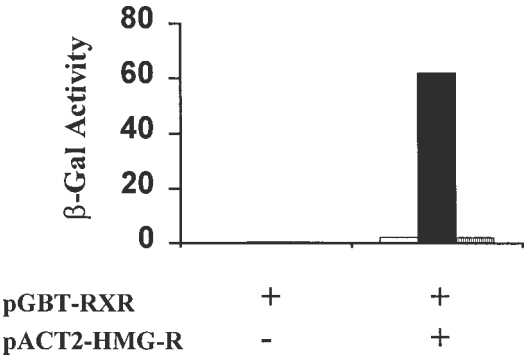

Fig. 2. Ligand-dependent interaction between *RXR* and *HMG-R*. Yeast cells were cotransformed with pGBT-*RXR*α and pACT2-*HMG-R*. β-galactosidase activity, indicating interaction between *RXR* and *HMG-R* in the absence of any ligand (open bar) or presence of *RXR*-specific ligand, AGN 194204 (1 μ*M*) (closed bar) or in the presence of an *RAR*-specific ligand, TTNPB (1 μ*M* shaded bar), is shown.

Similarly, yeast cells transformed with both the retinoid X receptor (*RXR*) bait vector (pAS2*RXR*α) and the fish vector, pACT2-*HMG-R*, show a robust induction of β-Gal reporter activity only in the presence of an *RXR*-specific ligand but not an *RAR*-specific ligand (**Fig. 2**). Therefore, an *RXR–HMG-R* interaction assay can be used for the identification of *RXR* selective, as well as for *RXR* subtype selective compounds.

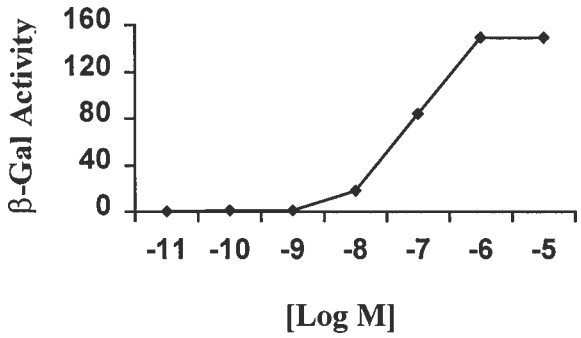

Fig. 3. Dose-dependent interaction between *RAR*γ and *HMG-R*. Yeast cells were cotransformed with pAS2*RAR*γ and pACT-*HMG-R*. β-galactosidase activity, indicating interaction between *RAR* and *HMG-R* in the presence of various concentrations of an *RAR*-specific ligand, TTNPB is shown.

3.8.1.2. Dose-Dependent Interaction Between *RAR* and *HMG-I*

The potency of a retinoid in the induction of *RAR–HMG* interaction can only be quantitated by a dose response curve. The *RAR–HMG-I*/R interaction is dose-dependent as shown in **Fig. 3**. The EC_{50} value (dose of the retinoid required for 50% induction of β-Gal activity) for TTNPB for *RAR*γ*-HMG-R* interaction is 70 n*M*, which is similar to its EC50 value in transactivation assays through *RAR*γ and an *RA*-responsive reporter ***(17)***. Yeast cells in **Fig. 3** are cotransformed with wild-type *RAR*γ expression vector (pAS2*RAR*γ) and pACT2-*HMG-R*. Similar results are obtained with pAS2*RAR*γ and pACT2-*HMG-I* transformed yeast cells (data not shown). The dose-dependent interaction was also observed between the *RAR*ΔAB constructs and the *HMG-I*/R in yeast cells. Further, these results show that full-length receptor can also be used in these assays as a bait vector.

3.8.2. RAR–RIP140 *Interaction*

RIP140 is a cofactor for *ER* mediated transcription, and it interacts with *ER* in the presence of estradiol but not in the presence of anti-estrogens ***(2)***. The ligand-dependent sequestration of *RIP140* by *RAR* appears to be a plausible mechanism for negative regulation of *ER*-responsive genes and for the anti-estrogenic effects of retinoids on breast cancer cells in vitro and in vivo. *ER* may be only one of the enhancer factors, which requires *RIP140* for their activity, and there may be other transcriptional pathways dependent on *RIP140*. *RAR–RIP140* interaction would thus disrupt those *RIP140*-dependent signal transduction pathways as well. We have recently shown that two distinct domains of *RIP140*, namely RID1 and RID2, independently interact with *RAR*γ

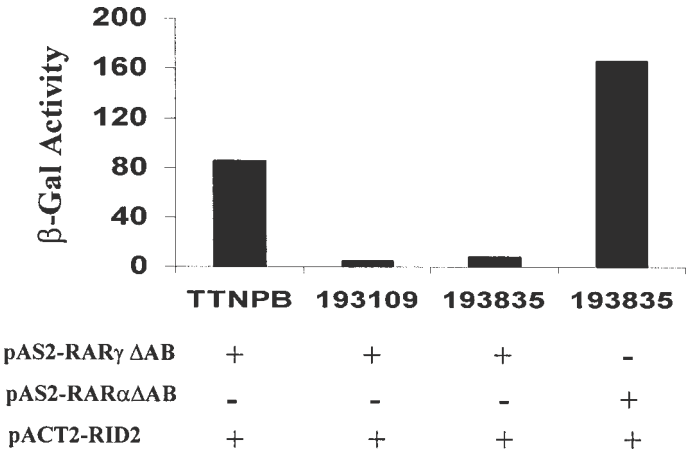

Fig. 4. Agonist-dependent interaction between *RAR* and RID2. Yeast cells were cotransformed with pAS2-*RAR*γΔAB or pAS2-*RAR*αΔAB, along with pACT-RID2. β-galactosidase activity of transformed yeast cells grown in the presence of various ligands (1 μ*M* each) is presented.

in a ligand dependent manner. Further, RID2 interacts with all three *RAR* subtypes, and the authors have developed an *RAR*α–RID2 interaction assay for the identification of retinoids of potential use in breast cancer. The therapeutic importance of *RAR*α*–RIP140* interaction is emphasized by a positive correlation between the ability of retinoids to inhibit the growth of breast cancer cells in vitro and to facilitate *RAR*α–RID2 interaction in vivo in yeast. Thus, retinoid ligands, which are potent in mediating *RAR*α*–RIP140* interaction, may exhibit better therapeutic indices for the treatment of breast cancer and uterine hyperplasia in postmenopausal women.

3.8.2.1. Ligand-Dependent Interaction Between RID2 and *RARs*

Cotransform yeast cells with pAS2*RAR*αΔAB, pAS2*RAR*βΔAB or pAS2*RAR*γΔAB and pACT2–RID2 expression vectors. Select the transformants on SD-Leu-Trp medium agar plates. Subject yeast colonies to liquid β-Gal assay in the presence or absence of the retinoid TTNPB or another synthetic compound whose receptor specificities need to be determined. As shown in **Fig. 4**, in the experiments involving transformation of yeast cells with pAS2*RAR*γΔAB and pACT2–RID2, TTNPB, being a pan-*RAR* agonist, facilitates interaction between *RAR*γ and RID2, but an *RAR* antagonist/inverse agonist, AGN 193109 ***(34)***, does not induce *RAR*γ-RID2 interaction. Further, an *RAR*α-specific retinoid, AGN 193835, does not induce *RAR*γ–RID2 interaction, but results in *RAR*α–RID2 interaction (**Fig. 4**). These results demonstrate

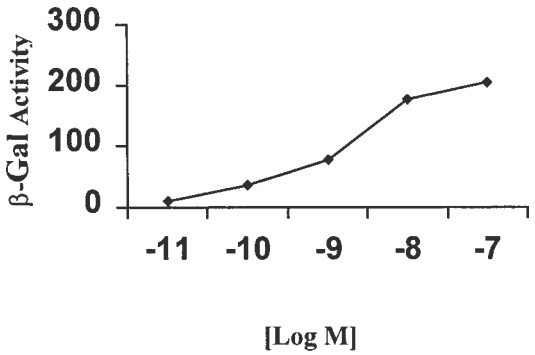

Fig. 5. Effect of tazarotene on *RAR*α–RID2 interaction. Yeast cells were cotransformed with pAS2-*RAR*αΔAB and pACT2-RID2 and β-galactosidase activity in the presence of various concentrations of tazarotene, an *RAR*β/γ-selective retinoid, is shown.

that an *RAR*–RID2 interaction assay in yeast can be used for the identification of *RAR* agonists, antagonists as well as receptor subtype selective retinoids.

3.8.2.2. *RAR*-RID2 Interaction for Identification of *RAR* Function-Selective Retinoids

Retinoids, which are potent inhibitors of T47D breast cancer cell growth, are also potent inducers of *RAR*α–RID2 interaction in yeast. Therefore, an *RAR*α–RID2 interaction assay can be used to identify function-selective retinoids. Further, it can also identify retinoids that are dissociated in their *RAR*α-dependent transactivation and *RAR*α–RID2 interaction properties. Tazarotene, an *RAR*β/γ-selective retinoid, is currently being developed for the treatment of breast and other cancers. This retinoid, although *RAR*β/γ-selective in transactivation assays, is equipotent to TTNPB, an *RAR* pan-agonist, in *RAR*α–RID2 interaction assays as well as in inhibiting the growth of breast cancer cells (**Fig. 5**). In **Fig. 5**, yeast cells are transformed with pAS2*RAR*αΔAB and pACT2-RID2 expression vectors and the transformants were analyzed for β-Gal activity in the absence or presence of various concentrations of ligands. The EC_{50} for tazarotenic acid (the free acid active form of tazarotene), in mediating *RAR*α–RID2 interaction and inhibition of breast cancer cell growth is 2–10 n*M*. Its EC_{50} value for the induction of gene expression in transient transfection assays through *RAR*α is >1000 n*M* ***(17)***. This class of retinoids, which facilitates only *RAR*α–RID2 interaction but do not transactivate through *RAR*α, may exhibit a better therapeutic index in the treatment of breast cancer.

4. Notes

1. Make sure by DNA sequencing that the bait construct contains the gene of interest in frame with the GAL4 DBD. Transform into yeast cells as described in **Subheading 3.2.** and confirm by Western blot analysis that the fusion protein of the proper size is produced in yeast. If specific antisera are not available, then perform Western blotting with anti-HA antibodies. The bait construct also has an in-frame HA-tag.
2. Disperse yeast clumps by thorough vortexing. The presence of clumps in the overnight-grown cultures would result in slower growth of yeast cells.
3. Do not amplify the fish cDNA library by growing the transformed bacteria in liquid culture. Always perform amplification in semisolid medium. This procedure minimizes the representational biases that can occur during the amplification of cDNA libraries.
4. Always grow the bait construct in SD-Trp medium to keep selective pressure on the extrachromosomal plasmid.
5. Keep SD-agar plates at RT for 2–3 d, or at 30°C for 3 h before plating the transformation reaction. This exercise will evaporate the excess moisture, which may interfere with even spreading of the transformation mix.
6. Because transcription factors are the bait constructs, they may induce reporter gene transcription, even in the absence of any fish construct vector. Thus, transformants carrying the bait construct alone may exhibit growth on SD-His plates. This leaky low level of *HIS3* gene expression is avoided by the addition of 25 m*M* 3-AT into the medium. Typically, 25–50 m*M* 3-AT is sufficient to stop the leaky expression of the reporter genes. Because 3-AT is heat-labile, do not add its stock solution to media hotter than 55°C. 3-AT is a competitive inhibitor of HIS3 protein.
7. Add the retinoid TTNPB to media after cooling it to 50–55°C.
8. Do not use nitrocellulose filter papers. They will crack when frozen with liquid nitrogen.
9. Prepare fresh 4 mg/mL ONPG solution every time a liquid β-Gal assay is performed.
10. The time taken for the appearance of yellow color will depend on the strength of interaction between the bait and the fish proteins. Stronger interactions will be visible in minutes, but weaker interactions may take hours (even sometimes 24 h) for the color to develop. The yellow color is not stable and becomes intense with time. Therefore, every experiment needs its own blank control for quantitating absorbence.
11. Make sure that the cell debris does not come with the supernatant: It will interfere with the spectrophotometer reading.
12. Yeast strain Y190 carries a cycloheximide resistant mutant allele, *cyh*^{r}*2*. The wild type cycloheximide sensitive (*cyh*^{s}*2*) allele is dominant to the mutant-resistant allele. PAS2 contains a *cyh*^{s}*2* gene, which would render Y190 yeast sensitive to cycloheximide treatment. Therefore, yeast cells that have spontaneously lost the pAS2 plasmid but still contain the pACT2 plasmid can be easily selected by

plating the cotransformants on SD-Leu cycloheximide medium plates. Cycloheximide is heat-labile; therefore, it should be added after cooling the medium to 55°C. Cycloheximide-containing plates can be stored for a period of 1 mo at 4°C.

13. In cotransformation experiments, use 0.5 μg each of the bait and the fish plasmids along with 10 μg carrier DNA.

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