

Enzymes used in Recombinant DNA Technology

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Lesson: Enzymes used in Recombinant DNA Technology

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Enzymes used in Recombinant DNA Technology

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Introduction

Recombinant DNA technology includes the procedures for creating recombinant DNA (rDNA). rDNA is a recombinant molecule where the vector is joined with a natural or synthetic DNA segment of interest to make a molecule that can replicate in a living cell. To produce rDNA one must be able to cut the vector at precise sites so that the DNA of interest can be inserted. The digested vector molecule and the DNA of interest are joined together to form a rDNA molecule. In the last few years, many new technologies for the manipulation of DNA have been developed. These techniques allow not only cutting and joining of DNA, but also shortening, lengthening and amplification of DNA molecules, copying into RNA and also development of new and modified DNA molecules by the addition or removal of specific chemical groups. These manipulations have not only led to genetic engineering but also increased our knowledge about gene structure and control of gene expression.

An important component of making rDNA is that most of the DNA manipulations are done *in vitro*, i.e., outside the living cells. This requires simulation of conditions and apparatus present in a living cell, in a test tube. In a living cell, enzymes participate in all crucial processes involved in DNA modifications. These processes include DNA replication, transcription, repair of mutated and damaged DNA, recombination between different DNA molecules and breakdown of unwanted or foreign DNA (for example invading viral DNA). If these enzymes can be isolated and purified, the above said processes can be done in a test tube (artificial conditions), provided that all catalytic requirements of the enzymes are fulfilled. Purified and high quality DNA modifying enzymes hold the key to the essence of genetic engineering. This has resulted in development of a big industry involved in production and supply of purified DNA modifying enzymes targeting the molecular biologists.

In this chapter, we will try to have a glance at the variety of DNA modifying enzymes available to the molecular biologists. Special emphasis will be given to enzyme responsible for precise cutting of the DNA molecules called as 'restriction endonuclease'.

Variety of DNA Modifying Enzymes

DNA recombinant technology requires a whole toolkit for modifying/manipulating DNA. These tools invariably include a variety of enzymes comprehensively called as DNA modifying enzymes. These include –

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1. Nucleases
2. Ligases
3. Polymerases
4. DNA modifying enzymes

Most of the reactions performed by the abovesaid enzymes cannot be accomplished by non-enzymatic chemical methods, hence underlining their importance in molecular cloning. The above said classes of enzymes differ in the reactions they catalyse, but some enzymes may perform more than one reaction. Also, the enzymes mentioned here are useful only for DNA manipulation but other similar enzymes modifying RNA are also available.

Nucleases

Nucleases are enzymes that degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next in a DNA strand. Nucleases can be broadly categorized into

- (i) exonucleases and (ii) endonucleases.

Exonuclease removes the terminal nucleotide of the DNA molecule by breaking the phosphodiester bond, whereas endonuclease breaks the internal phosphodiester bond.

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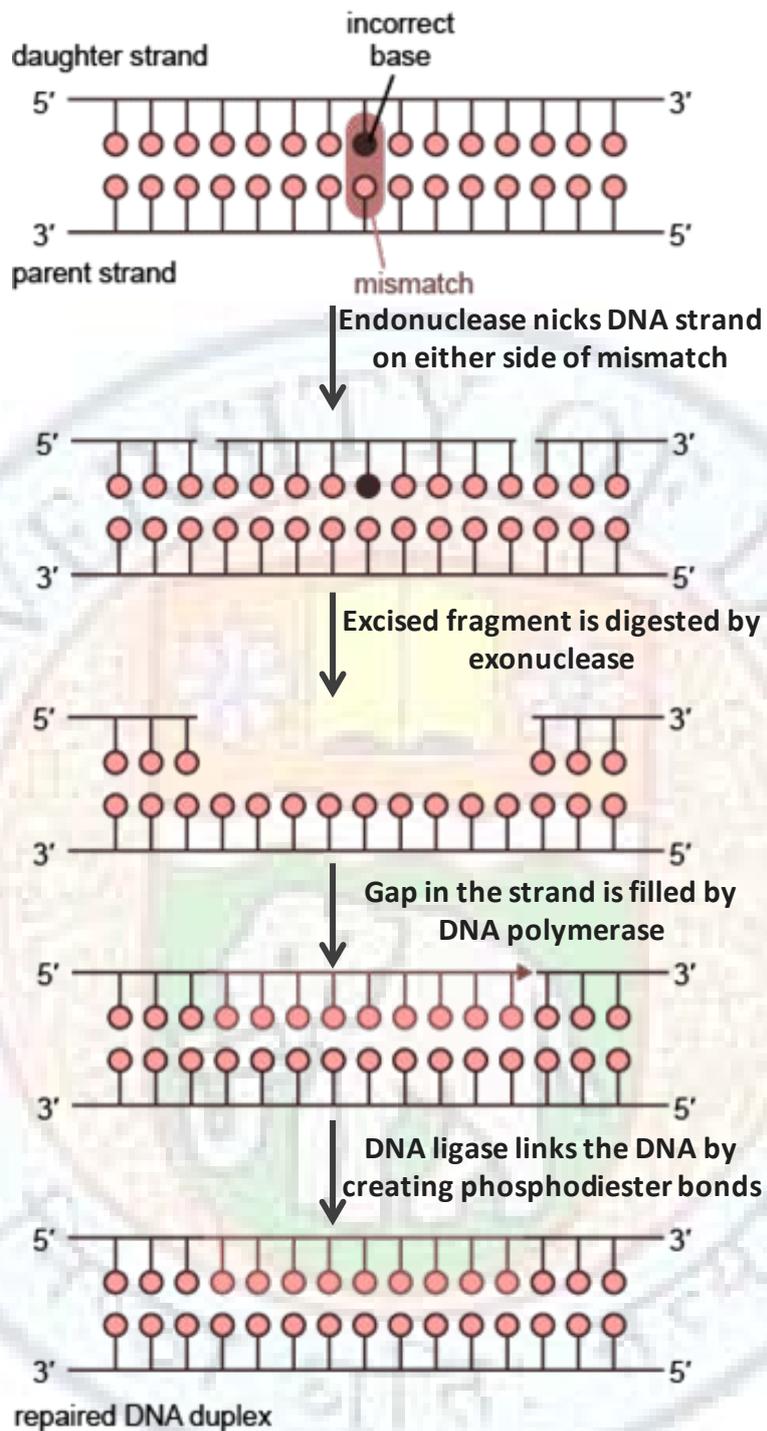


Figure:Diagrammatic representation of mismatch repair of DNA showing difference in the mode of action of endonuclease and exonuclease.

Source: Ms. Manisha Sharma

Different types of exonucleases can be categorised on the basis of number of strands they degrade in a double stranded DNA molecule. An exonuclease named Bal31 is

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isolated from a marine bacterium *Alteromonas espejiana*. It is a Ca^{2+} dependent enzyme that degrades the nucleotides from both the strands of dsDNA molecule. The longer the DNA is incubated with Bal31, the shorter the DNA molecule becomes. On the contrary, an enzyme isolated from *E. coli* called exonuclease III digests only one strand of the dsDNA molecule. It removes the nucleotide from the 3' terminus of the strand, thus leaving protruding 5' overhangs. Exonuclease III is used for generating single stranded templates.

Types of Exonucleases

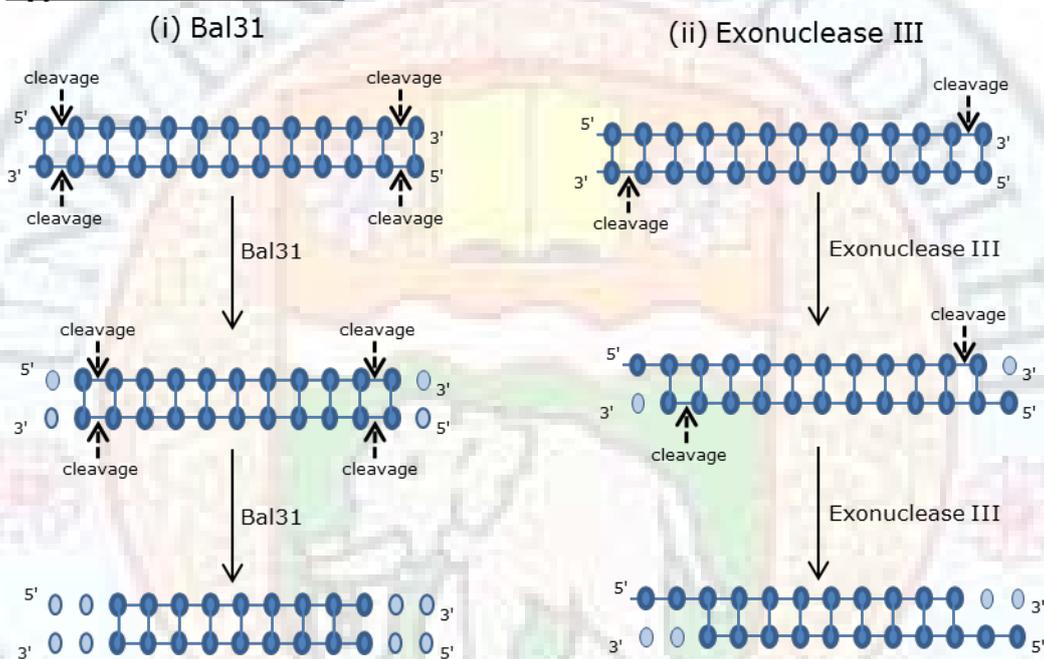


Figure: Diagrammatic representation of reactions catalysed by different types of exonucleases.

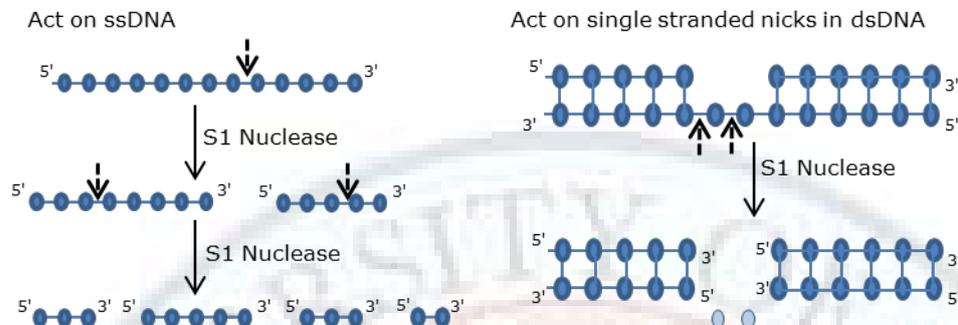
Source: Author

Similar to exonucleases, endonucleases can also be categorised based on whether they act on single or double stranded DNA. S1 nuclease is an endonuclease that is isolated from the fungus *Aspergillus oryzae*. It is a heat stable enzyme that functions at high ionic strength, low pH and in the presence of Zn^{2+} ions. It cleaves only single stranded DNA. Also, it is able to cleave the single stranded nicks in dsDNA molecules. Another type of endonuclease called as DNase I that is isolated from cow's pancreas is a non-specific enzymes. It is able to cleave both single and double stranded DNAs. It can cleave any of the internal phosphodiester bonds, thus prolonged digestion of DNA with DNase I results in its complete chewing leaving only a mixture of mononucleotides.

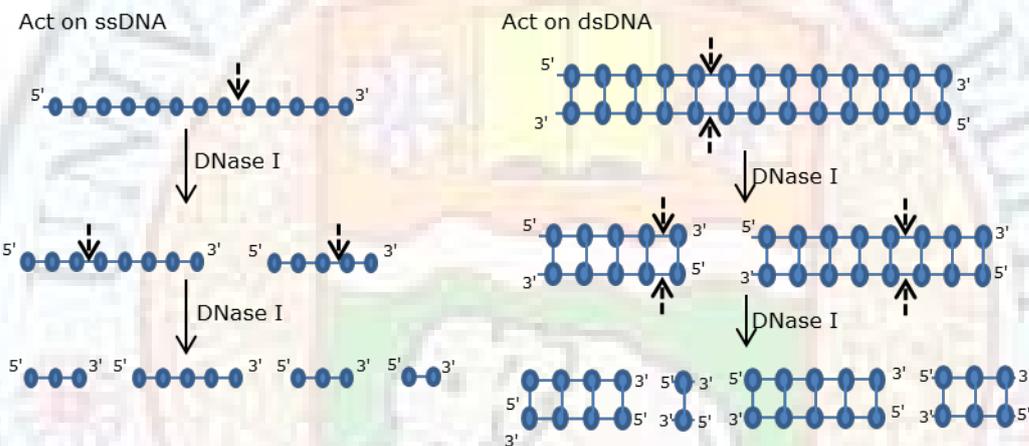
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Types of Endonucleases

(i) S1 Nuclease



(ii) DNase I



(iii) Restriction Endonuclease

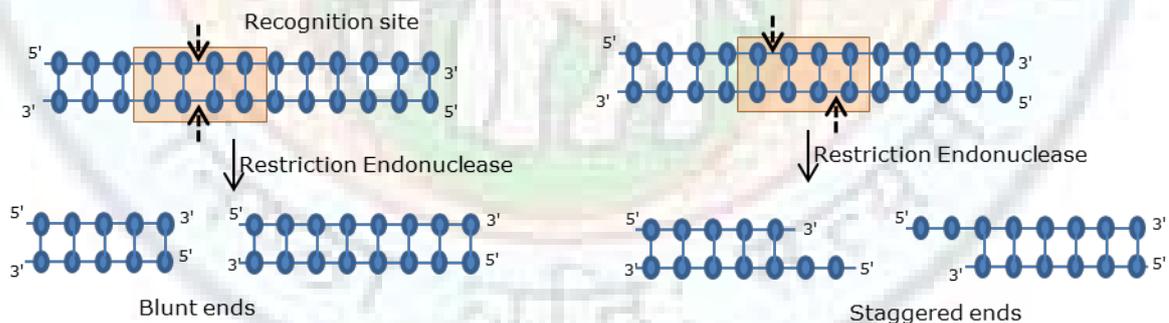


Figure: Diagrammatic representation of reactions catalysed by different types of endonucleases.

Source: Author

Another class of endonucleases are called as restriction endonucleases. The cleavage of DNA by these enzymes is very specific at 'particular sites'. Specific order of nucleotide

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sequences are recognized by restriction enzymes on the DNA that are then cleaved. Two kinds of ends (blunt or staggered) may be formed due to digestion of DNA by different kinds of restriction enzymes. This special class of endonucleases are dealt with in detail in the later part of this chapter.

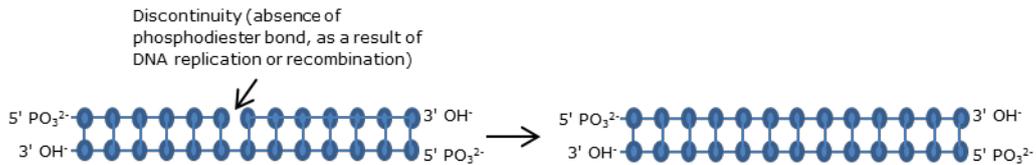
Ligases

Ligases are enzymes that join the nucleic acid molecules together. These nucleic acids can either be DNA or RNA, and the enzymes are thus called DNA ligase and RNA ligase, respectively. DNA ligase catalyses the formation of a phosphodiester bond between the 5' phosphate of one strand and the 3' hydroxyl group of another. In nature the function of DNA ligase is to repair single strand breaks (discontinuities) that arise as a result of DNA replication and/or recombination. In recombinant DNA technology, ligases catalyse the joining of DNA of interest called as 'insert', with the vector molecule and the reaction is known as ligation.



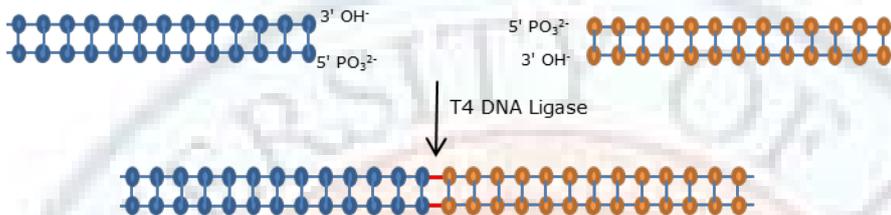
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Role of DNA Ligase in nature (to repair the discontinuities in DNA)



Role of DNA Ligase in genetic engineering (to join two DNA molecules)

Blunt end ligation



Sticky end ligation

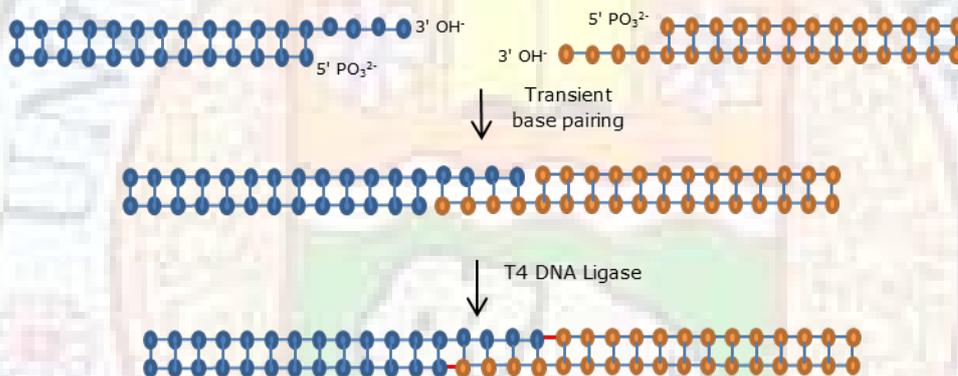


Figure:Diagrammatic depiction of reactions catalysed by DNA ligase. In nature they repair the missing phosphodiester bonds in one strand of DNA. In recombinant DNA technology, they are used to join two DNA molecules.

Source: Author

The discovery of DNA ligases was equally important as that of restriction endonucleases in the development of recombinant DNA technology. In 1967, Marty Gellert identified an enzyme that could form phosphodiester bonds between apposing 3' hydroxyl end and 5' phosphoryl end in a strand of DNA held in a double stranded configuration. This discovery allowed the molecular biologists to join different DNA molecules to form recombinant molecules.

For molecular cloning, the most commonly used DNA ligase is obtained from bacteriophage T4. T4 DNA ligase requires ATP as cofactor and Mg²⁺ ions for its activity. It is able to perform blunt end as well as sticky end ligations. In blunt end ligation, two

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phosphodiester bonds are formed between the two DNA molecules without any previous base pairing. This reduces the efficiency of blunt end ligation because it is difficult to hold the two DNA molecules in close proximity to affect the catalysis. On the other hand, ligation of two cohesive termini is highly facilitated by transient base pairing.

Polymerases

DNA polymerases are enzymes that catalyse the synthesis of a new DNA strand from a pre-existing strand. The enzyme adds deoxyribonucleotides to the free 3'-OH of the chain undergoing elongation. The direction of synthesis is 5'-3'. It has three major requirements for its activity; (1) a template strand for which the enzyme synthesizes a complementary strand; (2) a primer with a free 3'-OH group that hybridizes with the template to form a double stranded region that initiates the polymerization and (3) a pool of all the four dNTPs that are used to synthesize the new DNA strand. In addition, some cofactors like Mg^{2+} ions may be required in a buffer solution with correct pH for optimum activity.

A typical polymerase reaction

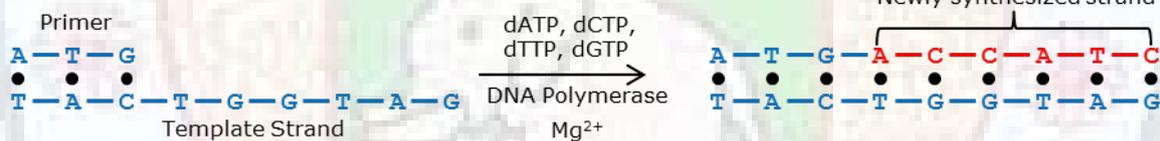


Figure: A typical reaction catalysed by DNA polymerase.

Source: Author

Different types of DNA polymerases are used in recombinant DNA technology. We will study the following types in detail.

1. *E. coli* DNA Polymerase I
2. Klenow Fragment
3. Thermostable DNA Polymerase
4. Reverse Transcriptase

E. coli DNA polymerase I (PolI) is an enzyme that has both DNA polymerase as well as DNA nuclease activity. This enzyme binds to the 'nick' region (region of a double stranded DNA where one or more nucleotides of one strand are missing, making it single

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stranded). The polymerase activity of the enzyme synthesizes the complementary strand for the nick and continues synthesizing the complete new strand by simultaneously degrading the pre-existing strand by its 5'-3'exonuclease activity.

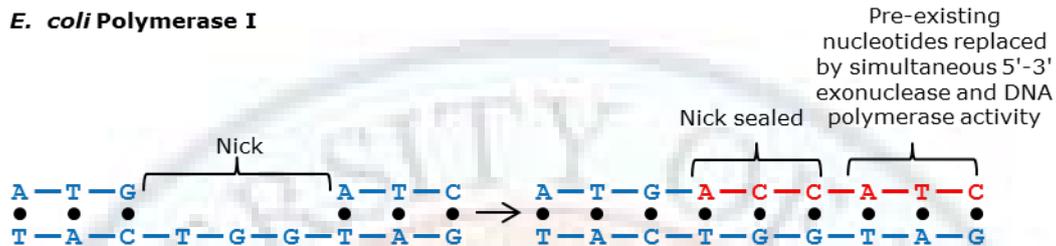


Figure: Reaction catalysed by *E. coli* DNA polymerase I.

Source: Author

Different domains of the *E. coli* Pol I are responsible for different catalytic activities. The C-terminal is responsible for the polymerase activity whereas the N-terminal of the enzyme catalyses the 5'-3'exonuclease activity. The central region of the enzyme is responsible for 3'-5' exonuclease activity that can remove any misread nucleotide and hence acts as a proofreading mechanism. The functions of different domains of *E. coli* Pol I are summarized in the table given below.

Table: Functions of different domains of *E. coli* Pol I.

Functions of different catalytic domains of *E. coli* Pol I

Domain	Catalytic Activity	Function
C-Terminal Domain	5'-3' Polymerase	Addition of nucleotides to 3'hydroxyl termini of RNA/DNA.
Central Domain	3'-5' Exonuclease	Cleavage of nucleotides from the 3'end.
N-Terminal Domain	5'-3'Exonuclease	Cleavage of nucleotides from the 5'end.

Source: author

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If the *E. coli* Pol I holoenzyme is treated with a mild protease, it results in the formation of two fragments. A larger fragment retaining both 5'-3' polymerase and 3'-5' exonuclease activities; while the smaller one has only the 5'-3' exonuclease activity. The larger fragment is known as 'Klenow fragment'.

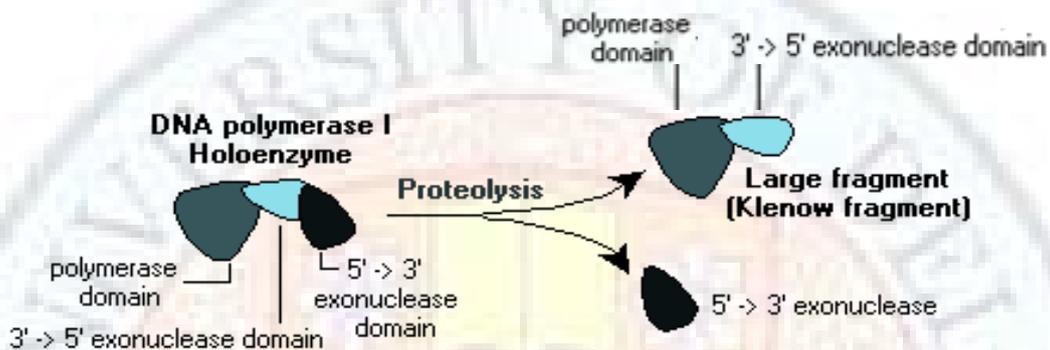
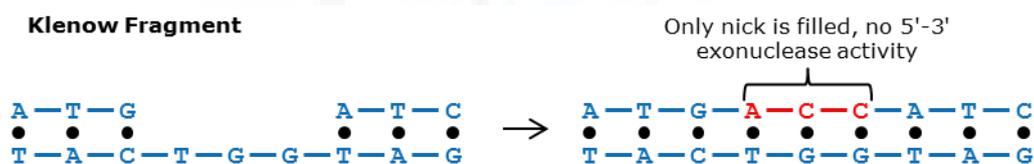


Figure: Diagrammatic representation of proteolytic cleavage of *E. coli* Pol I holoenzyme into two fragments having different catalytic activities.

Source: Ms. Manisha Sharma

This Klenow fragment can synthesize the new DNA strand complementary to the template but cannot degrade the existing strand. Klenow fragment is predominantly used in DNA sequencing. Other uses in recombinant DNA technology where Klenow fragment is used are

- Synthesis of double stranded DNA from single stranded template.
- Filling of 5' overhangs created by restriction enzymes to create blunt ends.
- Digestion of protruding 3' overhangs to produce blunt ends.



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Figure: Diagrammatic representation of reaction catalyzed by reverse transcriptase. Note that the initial template is a RNA and not DNA.

Source: Author

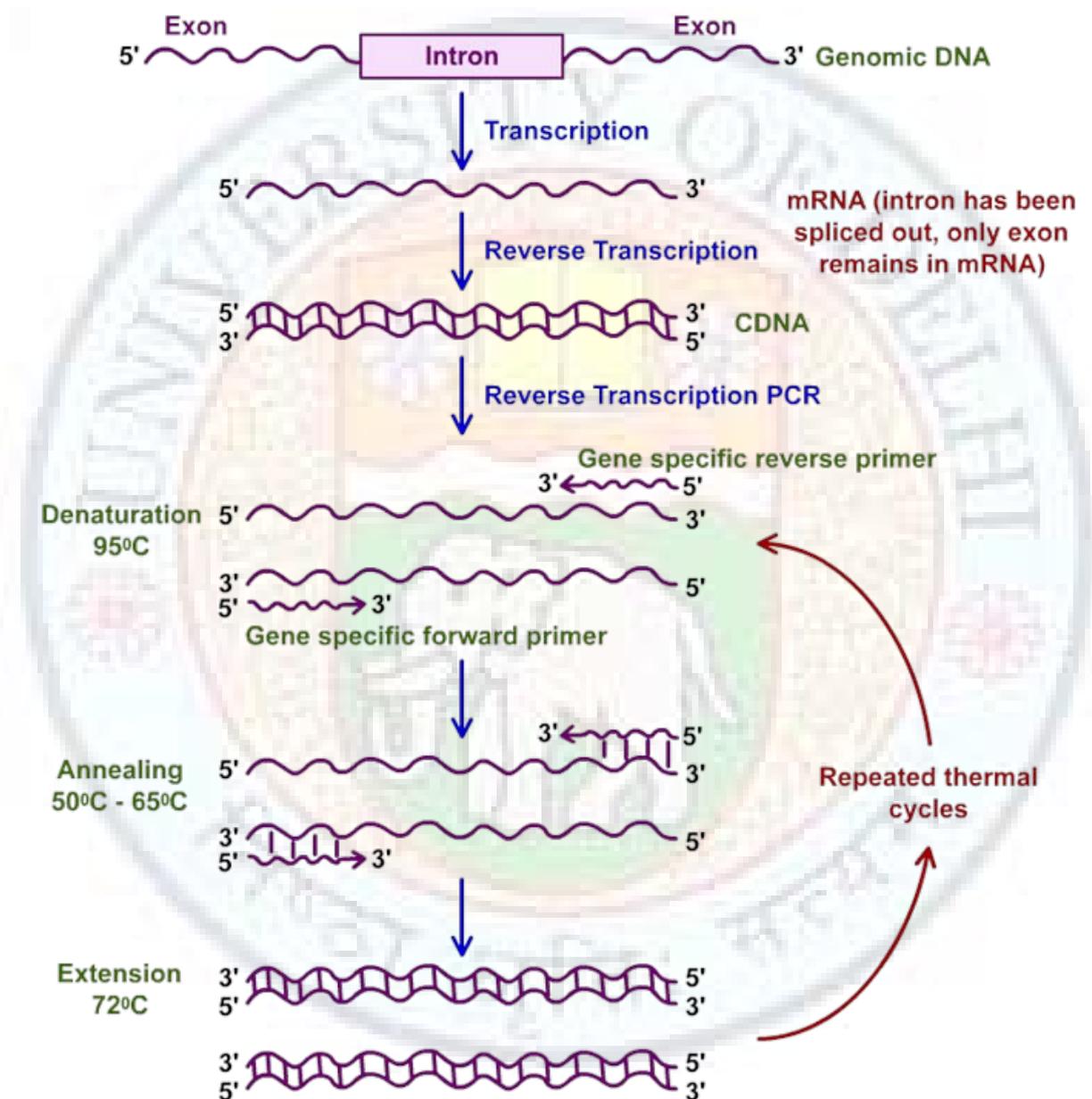


Figure: Diagrammatic representation showing reverse transcriptase PCR.

Source: Ms. Manisha Sharma

DNA modifying enzymes

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Addition or deletion of different chemical groups from DNA molecules comes in handy for molecular cloning purposes. Three such enzymes known for performing such modifications are:

1. **Alkaline phosphatase (AP):** This group of enzymes removes the phosphate group (PO_3^{2-}) from 5' terminus of the DNA molecule. It is active at alkaline pH, hence the name 'alkaline phosphatase'. Commercially, it is obtained from three major sources, viz., *E. coli* (bacteria), calf intestine and arctic shrimp.

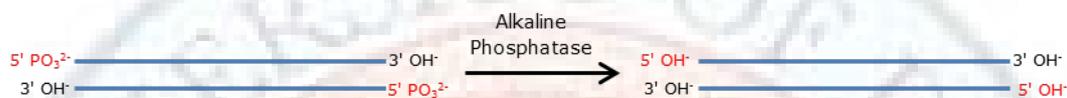


Figure: Diagrammatic representation of reaction catalyzed by alkaline phosphatase.

Source: Author

Treatment of vector DNA with AP is important in cloning experiments, as removal of 5' phosphate prevents self-annealing of the digested vector and increases the possibility of ligating with the insert DNA fragment in the presence of ligase. Also, radiolabeled DNA probes are prepared by initially removing the $5'\text{PO}_3^{2-}$ by AP treatment, followed by polynucleotide kinase treatment in the presence of radioactive phosphate.

2. **Polynucleotide kinase (PNK):** This group of enzymes perform a role completely opposite to the one performed by AP. PNK catalyses the transfer of a phosphate group from ATP to the 5' terminus of the DNA molecule. This enzyme is obtained from *E. coli* infected with T4 phage.

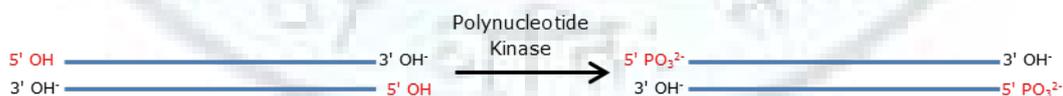


Figure: Diagrammatic representation of reaction catalysed by polynucleotide kinase.

Source: Author

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- 3. Terminal transferase:** This group of enzymes catalyses the addition of one or more deoxyribonucleotides to the 3' terminus of the DNA molecule. The enzyme can work on both double stranded as well as single stranded DNA molecules without the need of any primers. The enzyme is obtained from calf thymus tissue.

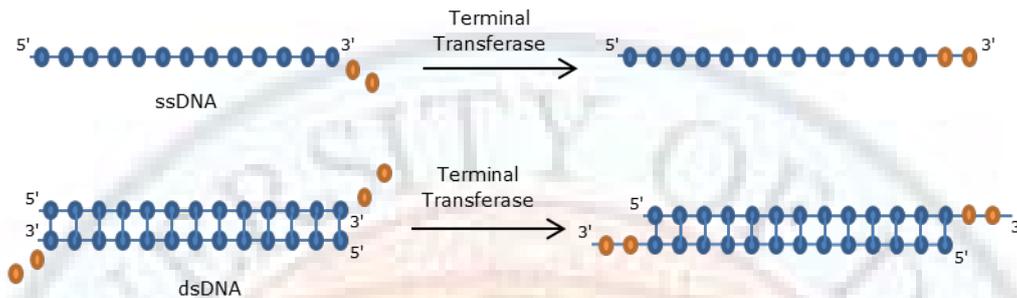
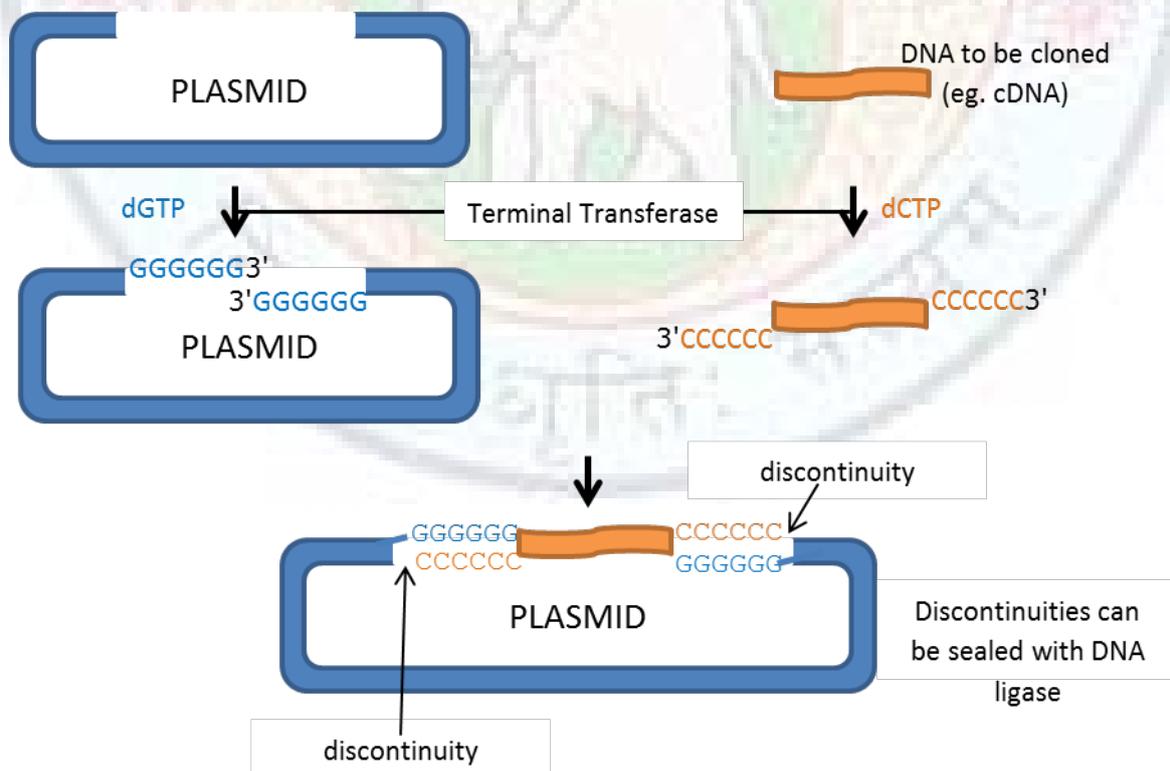


Figure:Diagrammatic representation of the reaction catalysed by terminal transferase.

Source: Author

The enzyme is used for labelling 3' ends of DNA. Also, it can be used for adding complementary homopolymeric tails to DNA molecules.



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Figure: Applications of terminal transferase in creating recombinant DNA. Terminal transferase can add complementary homopolymeric chains at 3' ends of vector and insert. The two molecules are joined together due to transient base pairing and finally the discontinuities are sealed using DNA ligase.

Source: Author

Molecular Scissors: Restriction Endonucleases

The foundation of molecular cloning was laid with the discovery of restriction enzymes. For DNA cloning, the vehicle carrying the DNA i.e., the vector must be cleaved to open up the circle. It is absolutely essential that the cutting is very precise. If the vector is cut in a random fashion generating two or more fragments, the vector DNA becomes non-functional. Restriction endonucleases enable this precise cleavage and hence are also known as 'molecular scissors'.

Generally every cloning vector has a 'polylinker region' also called as 'multiple cloning site' that is comprised of many restriction sites. This enables the molecular biologists to choose different restriction enzymes for multiple cloning procedures. The discovery of restriction enzymes was a crucial step that has revolutionized the field of genetic engineering.

Discovery and Biological Function of Restriction Endonucleases

In the early 1950s, four separate laboratories, almost simultaneously discovered a phenomenon known as 'host controlled restriction'. According to this mechanism, some bacteriophages were not able to grow in some strains of bacteria. In other words, bacteriophages were restricted in some strains of bacteria. In 1969, Arber and Linn showed that immune bacteria contained an enzyme that degraded the viral DNA before it could replicate in the host and direct the synthesis of new viral particles. Arber suggested that there were specific sites in viral DNA that were targeted by the enzyme for degradation. The restrictive and degrading enzymes were called as 'restriction endonucleases' or just 'restriction enzymes'.

Further studies showed that the bacteria's own DNA is protected from the enzymatic degradation because these specific sites are chemically modified in it. Another group of enzymes known as 'methyl transferases' were identified that could transfer the methyl group to one or more nucleotides of the target site in bacteria, thus protecting it from self-degradation. These two phenomena, i.e., restriction of the viral genome by

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endonucleases and the modification of host genome by methyl transferases, are together known as the 'Restriction-Modification System'.

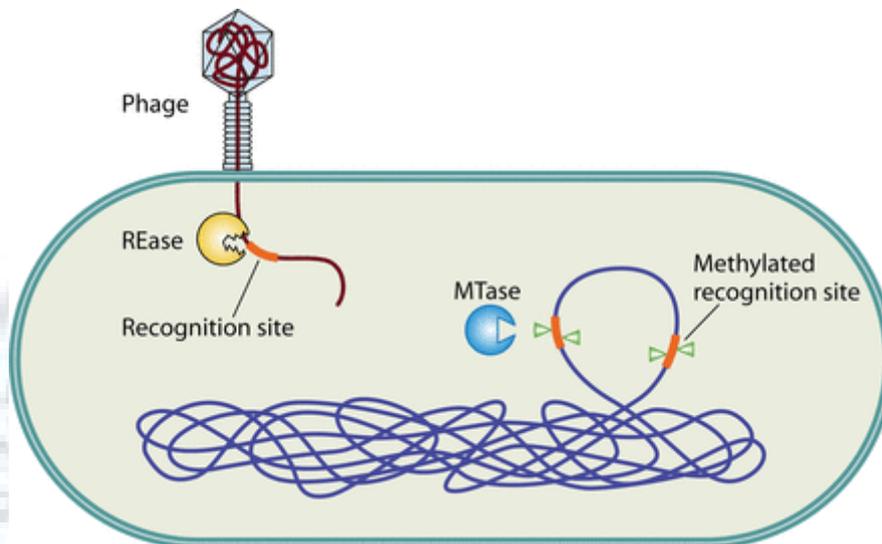


Figure: Illustration showing the Restriction-modification system as a defence mechanism of bacteria. Recognition sites present on bacterial DNA are methylated by methyl transferases (MTase), thus recognised as self and not acted upon by restriction endonucleases (REase). The phage genome lacking methylation of restriction sites is identified as non-self and thus cleaved by REases.

Source: <http://mmbbr.asm.org/content/77/1/53.full>

In 1978, Nathan, Arber and Smith won the Nobel Prize for the discovery of restriction enzymes and their applications to solving the problems of molecular genetics. They were among the first to describe the potentials of restriction enzymes in molecular engineering.

In 1978, then ten year old Sylvia Arber, daughter of Werner Arber who has received Nobel Prize together with Hamilton Smith and Dan Nathans, beautifully described the biological role as well as applications of restriction enzymes, in a small write-up published in News and Views article in Nature Structural Biology (Konforti, 2000).

Table: Description of restriction enzymes by Sylvia Arber.

Enzymes used in Recombinant DNA Technology

Interesting Facts!!

"When I come to the laboratory of my father, I usually see some plates lying on the tables. These plates contain colonies of bacteria. These colonies remind me of a city with many inhabitants. In each bacterium there is a king. He is very long, but skinny. The king has many servants. They are thick and short, almost like balls. My father calls the king DNA, and the servants enzymes. The king is like a book, in which everything is noted on the work to be done by the servants. For us human beings these instructions of the king are a mystery.

My father has discovered a servant who serves as a pair of scissors. If a foreign king invades a bacterium, this servant can cut him in small fragments, but he does not do any harm to his own king. Clever people use the servant with the scissors to find out the secrets of the kings.

To do so, they collect many servants with scissors and put them onto a king, so that the king is cut into pieces. With the resulting little pieces it is much easier to investigate the secrets. For this reason my father received the Nobel Prize for the discovery of the servant with the scissors."

Source: <http://www.nature.com/scitable/content/The-servant-with-the-scissors-17346>

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Interview of Dr. Werner Arber, one of the recipients of Nobel Prize for the discovery of restriction enzymes .

Source:<https://www.youtube.com/watch?v=yF3ZeeF3P8c>

Types of Restriction Endonucleases

At present more than 2500 different restriction endonucleases have been identified that are isolated from a number of species of bacteria. More than 300 restriction enzymes are now available for use in the laboratory. These enzymes belong to three different classes of restriction endonucleases that can be distinguished from each other on the basis of their structure, cofactors required and features of their restriction and cleavage site.

Type I

Type I restriction enzymes have a complex structure with three different subunits (endonuclease, methyl transferase and recognition). These enzymes cut DNA at a random site, far (~1 kb) from the recognition sequence. Their restriction sites are also complex and discontinuous with spacers. They may have biological significance but are of very little practical value since the site where the DNA is going to be digested cannot be predicted.

Type II

Type II restriction endonucleases cleave DNA at very precise and defined positions close to or within the recognition sequence. These enzymes require magnesium (Mg^{2+}) ions as cofactors for their activity. They are smaller in size, in comparison to type I and type III enzymes and mostly bind to DNA as homodimers. The recognition site is mostly symmetric, showing two fold symmetry. Most of the type II enzymes cleave within the symmetric recognition sequence, e.g. *HhaI*, *HindIII* and *NotI*, but there are other classes also that cleave outside their recognition sequence. A few other type II restriction enzymes cleave on both sides of the recognition sequence releasing a small fragment.

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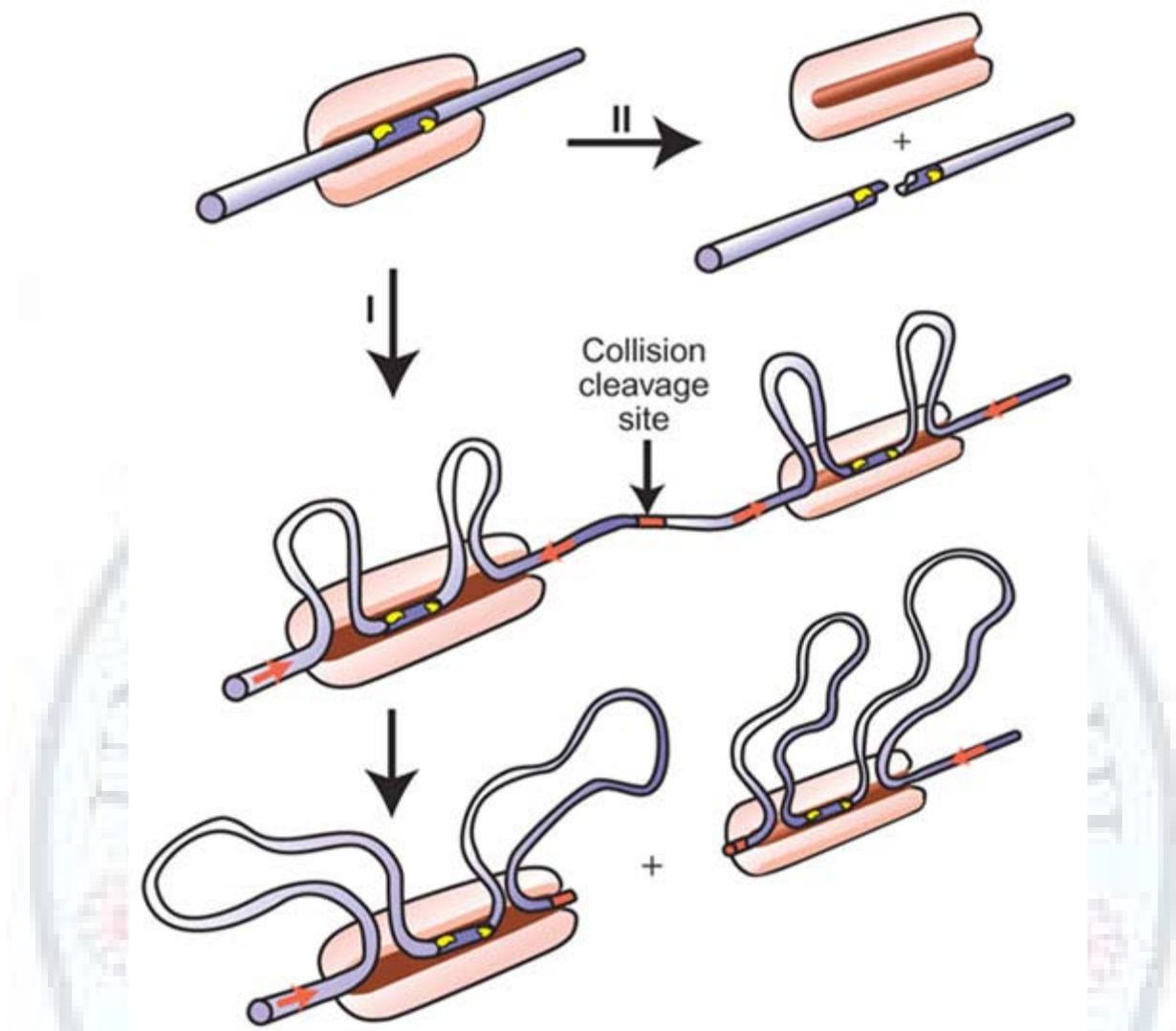


Figure: Diagrammatic representation showing difference in the action of type I and type II restriction enzymes. The specific target sequence (dark blue segment on the DNA shown as a tube) is recognized by the enzyme. A dimeric type II restriction enzyme cleaves the DNA at a specific location within or near the target sequence, and then dissociates from the DNA leaving two free ends. Type I restriction enzymes hold on to the target sequence and trigger DNA translocation (direction shown by red arrows). Contact between the DNA and enzyme is created at multiple points, so that DNA is extruded in loops as the enzyme keeps on reeling in DNA. Stalling of translocation by collision with, for example, another type I restriction enzyme, triggers cleavage at the collision site. After DNA cleavage, unlike the type II restriction enzymes, the type I restriction enzymes remain attached to the DNA and do not turn over in the restriction reaction.

Source: http://www.nature.com/nsmb/journal/v11/n9/fig_tab/nsmb0904-804_F1.html

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Type III

This class of enzymes are large and complex proteins that cleave outside the recognition sequence. The cleavage is non-specific and ~30 nucleotides away from the recognition sequence.

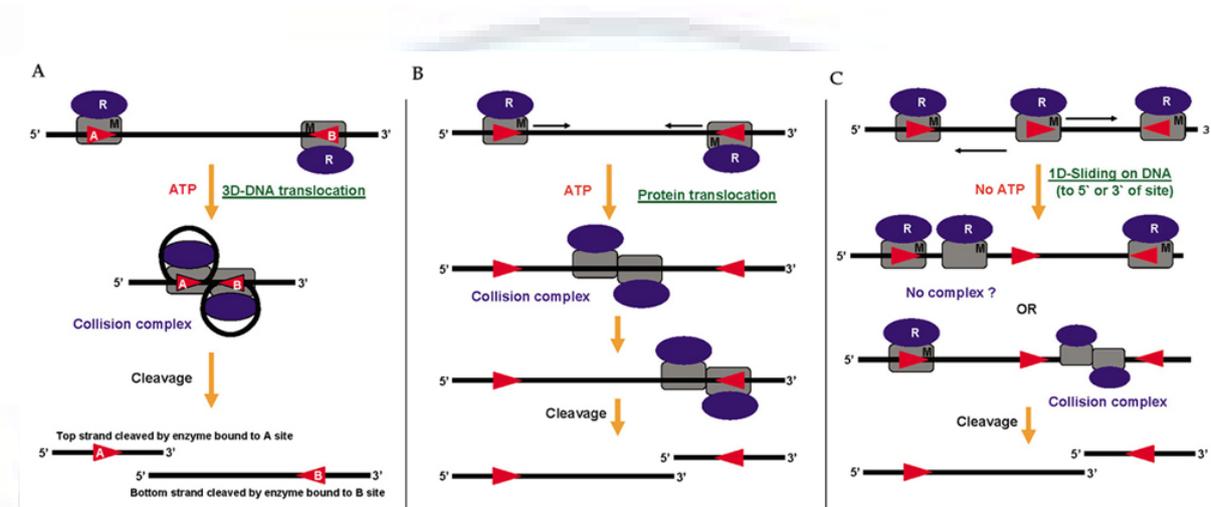


Figure: Schematic diagram showing three models proposed for the cleavage of DNA by type III restriction enzymes. A. DNA loop and translocation. B. Protein translocation. C. DNA sliding. Triangles A and B represent recognition sites for the restriction enzymes (R-M). The base of the triangle (5') is tail and tip (3') of the triangle is head of the recognition sequence. Orientation of restriction sites for different type III enzymes varies, and specific orientations are preferred as substrates by the enzymes. Recognition sites can be present in head-to-head, head-to-tail or tail-to-tail orientation.

Source: <http://www.bioscience.org/2012/v17/af/3975/fulltext.php?bframe=figures.htm>

Type I and Type III endonucleases are not of any experimental value in genetic engineering, but have biological significance. Type II endonucleases are the most widely used class of enzymes that are thoroughly exploited in various DNA analyses and gene cloning experiments. The following table summarizes and compares the three classes of restriction endonucleases.

Table: Comparison between type I, II and III restriction endonucleases.

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Characteristic features of different classes of restriction enzymes

Type of enzyme	DNA recognition sequence	Cleavage site	Example	Co-factor required	Experimental value
I	Asymmetric and complex; composed of two parts separated by a spacer	Non-specific; >1000 base pairs from recognition site	<i>EcoKI</i>	Mg ²⁺ , S-adenosyl methionine and ATP	Only biological significance
II	Two-fold rotational symmetry (Palindrome)	Specific; at the recognition site	<i>EcoRI</i> , <i>HindIII</i>	Mg ²⁺	In DNA analysis and molecular cloning
III	Short and asymmetric	Non-specific; 25-28 base pairs 3' to the recognition site	<i>EcoPI</i>	S-adenosyl methionine and ATP	Only biological significance

Source: author

Nomenclature of Restriction Endonucleases

At present, more than 3000 restriction endonucleases have been identified. Each has been provided a definite name. Enzyme nomenclature is based on the bacterium from which it was isolated. The first three letters of the enzyme name are derived from the first letter of the genus name and the first two letters of the species name. Since each bacterium may contain several different restriction enzymes, a Roman numeral is also used to identify each enzyme. In the case of enzymes isolated from different strains of the same bacterium, first letter of the strain is used before the Roman numeral. For example, *EcoRI* is derived from *Escherichia* (genus), *coli* (species), **R**Y1 (strain), **I** (first identified from bacterium). A few more derivations of some restriction enzyme names are given in the table below.

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Table: Derivation of restriction enzyme names.

Derivation of restriction enzyme names				
Enzyme name	Genus	Specific epithet	Strain	Order of identification
<i>EcoRI</i>	<i>E</i> <i>Escherichia</i>	<i>co</i> <i>coli</i>	R RY1	I First identified
<i>HindIII</i>	<i>H</i> <i>Haemophilus</i>	<i>in</i> <i>influenza</i>	d Rd	III Third enzyme identified
<i>PvuII</i>	<i>P</i> <i>Proteus</i>	<i>vu</i> <i>vulgaris</i>		II Second enzyme identified

Source: author

Features of recognition sequences and cleavage sites of type II restriction endonucleases

Each restriction enzyme recognizes a particular restriction site. This is a specific nucleotide sequence where the enzymes bind DNA. Each restriction enzyme cleaves phosphodiester bonds in DNA molecules at a specific cleavage site. In type II enzymes the cleavage site is within the recognition site. Following are some of the characteristic features of restriction and cleavage sites of type II enzymes.

Binding of enzyme to the recognition site: It is believed that the restriction enzyme binds to DNA and slides through it non-specifically, till it finds the correct recognition sequence. Recent evidences show that it is not just one dimensional sliding of the enzyme on the DNA molecule, but also two to three dimensional jumping and hopping of the DNA molecule that allows diffusion of the restriction enzyme to the required recognition site.

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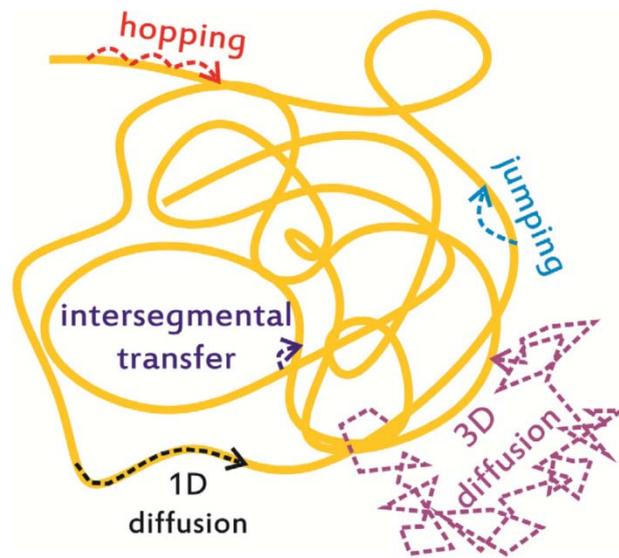


Figure: A model showing different mechanisms used by enzymes for searching the target site.

Source: <http://www.mdpi.com/1422-0067/14/2/3961/htm>

Symmetry: The recognition sequence for most of the type II restriction enzymes has an internal symmetry i.e. the same sequence can be read even after 180° rotation. Consider the following example of *EcoRI* recognition sequence.

5'—GAATTC--3'

3'—CTTAAG--5'

Here, 5' to 3' sequence of both the strands is same. A sequence with such two-fold rotational symmetry is called as a palindrome.

Length and frequency: Most recognition sequences are four to eight nucleotides long. Probability of finding a particular recognition site in a genome sequence is inversely proportional to the length of the recognition site. The frequency of occurrence of a particular recognition sequence in the genome decreases with increase in the length of the sequence. This is because, if we consider genome sequences to be random and percentages of the four nucleotides to be equal (=25%), then probability of finding a recognition site is once in $4^{\text{length of the recognition site}}$. Thus on average, a tetranucleotide recognition site would occur after every $4^4=256$ base pairs, whereas a hexanucleotide recognition site would occur after every $4^6=4096$ base pairs. Depending on the

Enzymes used in Recombinant DNA Technology

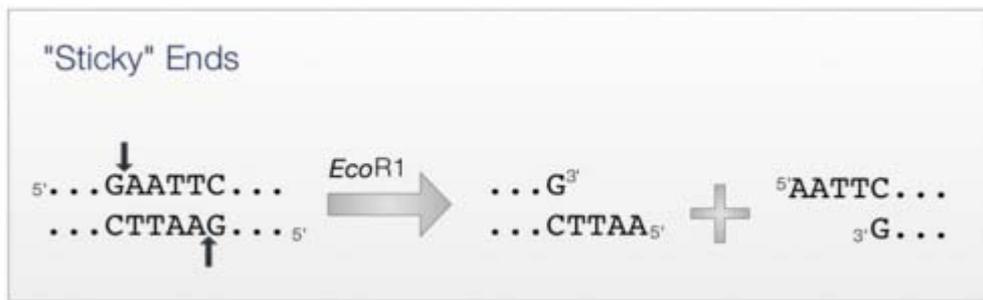
frequency of the recognition site, the restriction enzymes are categorized into 'rare cutters' and 'frequent cutters'.

In nature, the above said formula does not always work and the recognition sites are not evenly placed. The reason could be that all genomes do not have equal constitution of the four nucleotides. Also, the DNA sequence is not random ordered. Thus, despite the fact that mathematical calculations provide an idea about the frequency of recognition sites, one needs to actually perform restriction digestions to find it.

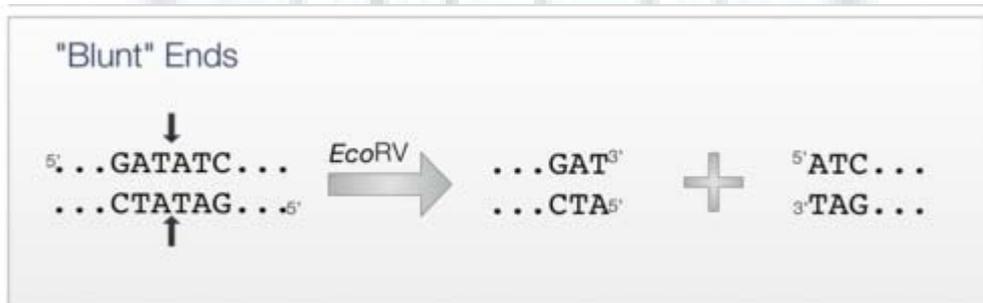
Blunt ends and sticky ends: There are principally two ways by which DNA is cut by restriction enzymes generating either a blunt cut or a staggered cut. The type of cut produced by the restriction enzyme determines the strategy of the cloning experiment. In a blunt cut, both the strands of the DNA are cut at the same place in the middle of the recognition site. This results in the production of 'blunt ends' or 'flush ends'. *Alu I*, *Hae III* and *Pvu II* are examples of blunt end cutters.

In a staggered cut, two strands of a dsDNA are not cut at the same position. Instead the two strands are cut at a position two to three nucleotides away from each other. This causes the formation of a small single stranded overhang. Such staggered cuts produce ends that are complementary to each other and stick back via transient base pairing. Thus, these ends are also called as 'sticky ends' or 'cohesive ends'. Joining back of sticky ends causes reconstitution of the recognition site. The overhangs produced by staggered cutters can be either '5' overhangs' or '3' overhangs'.

Enzymes used in Recombinant DNA Technology



A



B

Figure: Restriction enzymes recognize four to eight nucleotides long palindromic sequences in DNA and (A) make staggered cuts to generate sticky ends or (B) make straight cuts to generate blunt ends.

Source: <http://bio305lab.wikidot.com/project2014:enzymes-used> (cc)

Table: Some examples of type II restriction endonucleases.

Some of the most frequently used restriction endonucleases

Name of the enzyme	Organism	Sequence	Cuts Produced	Sticky or Blunt End
<i>AluI</i>	<i>Arthrobacter luteus</i>	5'--AGCT 3'--TCGA	5'---AG CT---3' 3'---TC GA---5'	Blunt
<i>HaeIII</i>	<i>Haemophilus aegyptius</i>	5'--GGCC 3'--CCGG	5'---GG CC---3' 3'---CC GG---5'	Blunt
<i>PvuII</i>	<i>Proteus vulgaris</i>	5'--CAGCTG 3'--GTCGAC	5'---CAGCTG---3' 3'---GTCGAC---5'	Blunt

Enzymes used in Recombinant DNA Technology

<i>EcoRI</i>	<i>Escherichia coli</i>	5'--GAATTC 3'--CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'	Sticky (5' overhang)
<i>BamHI</i>	<i>Bacillus amyloliquefaciens</i>	5'--GGATCC 3'--CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'	Sticky (5' overhang)
<i>BglII</i>	<i>Bacillus globigii</i>	5'--AGATCT 3'--TCTAGA	5'---AGATCT---3' 3'---TCTAGA---5'	Sticky (5' overhang)
<i>HindIII</i>	<i>Haemophilus influenzae Ra</i>	5'--AAGCTT 3'--TTCGAA	5'---AAGCTT---3' 3'---TTCGAA---5'	Sticky (5' overhang)
<i>NotI</i>	<i>Nocardia otitidis-caviarum</i>	5'--GCGGCCGC 3'--CGCCGGCG	5'---GC GGCCGC---3' 3'---CGCCGG CG---5'	Sticky (5' overhang)
<i>TaqI</i>	<i>Thermus aquaticus</i>	5'--TCGA 3'--AGCT	5'---TCGA---3' 3'---AGCT---5'	Sticky (5' overhang)
<i>HinfI</i>	<i>Haemophilus influenzae Rf</i>	5'--GANTC 3'--CTNAG	5'---G ANTC---3' 3'---CTNA G---5'	Sticky (5' overhang)
<i>Sau3A</i>	<i>Staphylococcus aureus</i>	5'--GATC 3'--CTAG	5'--- GATC---3' 3'---CTAG ---3'	Sticky (5' overhang)
<i>PvuI</i>	<i>Proteus vulgaris</i>	5'--CGATCG 3'--GCTAGC	5'---CGATCG---3' 3'---GCTAGC---5'	Sticky (3' overhang)
<i>Kpn I</i>	<i>Klebsiella pneumoniae</i>	5'--GGTACC 3'--CCATGG	5'---GGTAC C---3' 3'---C CATGG---3'	Sticky (3' overhang)
<i>Pst I</i>	<i>Providencia stuartii</i>	5'--CTGCAG 3'--GACGTC	5'---CTGCA G---3' 3'---G ACGTC---3'	Sticky (3' overhang)
<i>Sac I</i>	<i>Streptomyces achromogenes</i>	5'--GAGCTC 3'--CTCGAG	5'---GAGCT C---3' 3'---C TCGAG---3'	Sticky (3' overhang)

Source: author

Isoschizomers: Different enzymes identified from different bacteria may have the same recognition sequence, with the same cleavage site. Such restriction enzymes are known as isoschizomers. For example, *SphI* (CGTAC/G) and *BbuI* (CGTAC/G) are isoschizomers that recognize the same recognition sequence. If two enzymes have the same recognition site but different cleavage sites, they are known as neoschizomers. For example *SmaI* (CCC/GGG) and *XmaI* (C/CCGGG) are neoschizomers, where *SmaI* produces blunt ends, whereas *XmaI* produces cohesive ends. Some enzymes recognize similar (not same) sequences but produce the same sticky ends. Such enzymes are known as isocaudomers. For example, *NotI* and *Bsp120I*

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NotI : 5'-GC↓GGCC GC-3'
3'-CG CCGG↑CG-5'

Bsp120I : 5'-G↓GGCC C-3'
3'-C CCGG↑G-5'

Here, both the enzymes produce GGCC tetranucleotide overhangs.

Restriction enzymes can be classified into two more classes:

Type IV: Recognition sites for these enzymes consist of modified DNA, usually methylated sequences. Examples are the McrBC and Mrr systems of *E. coli*.

Type V: These enzymes use guide RNAs to target specific non-palindromic sequences found in invading organisms, and cut DNA to generate fragments of variable lengths. An example is the cas9-gRNA complex from CRISPRs, which can be utilised for genetic engineering applications.

Restriction mapping

A 'restriction map' is a diagrammatic representation of profile of restriction enzyme sites in a piece of DNA. The piece of DNA can be either a linear DNA (genomic DNA) or a circular DNA (plasmid). The restriction map also depicts the relative distance of nucleotides between two adjacent restriction sites.

Generation of restriction map is the first step in characterizing an unknown DNA for its structural attributes. To construct a restriction map, a series of restriction digestions must be performed. Restriction endonucleases which are infrequent cutters/rare cutters are used for generating the maps, so that countable numbers of fragments are obtained. DNA fragments thus obtained are then separated by 'agarose gel electrophoresis' (see [this link](https://www.youtube.com/watch?v=6mQGNDnOyH8) to know more about gel electrophoresis <https://www.youtube.com/watch?v=6mQGNDnOyH8>). This provides information about the number of fragments obtained after each digestion. Also, comparison with the DNA ladder (size marker) tells about the size of the DNA fragments. Number of fragments obtained after the single digestion can tell about the number of restriction sites of a particular enzyme present in a piece of DNA under analysis.

For Linear DNA: if the number of restriction sites present are 'n', then the number of fragments formed after digestion are 'n+1'.

Enzymes used in Recombinant DNA Technology

For circular DNA: if the number of restriction sites present are 'n', then the number of fragments formed after digestion are 'n'.

Further, for determining the order and orientation of fragments/restriction sites, a series of double digestions are performed. Here, the DNA is cut by two restriction endonucleases at the same time. It might be possible to perform a double digestion in one step if both enzymes have similar requirements for pH, Mg^{2+} concentration, etc. Otherwise, the two digestions have to be carried out one after the other, adjusting the reaction mixture after the first digestion to provide a different set of conditions for the second enzyme. It is important to obtain complete digestion of the DNA with each of the enzymes used since partial digest will yield false fragments which will lead to confusion.

Let us try to analyse the following examples of linear and circular pieces of DNA digested with different restriction enzymes.

Example 1:

A linear DNA was digested with *EcoRI* and *HindII* separately. Double digestion using both the enzymes simultaneously was performed as well. Data for fragments obtained for each digestion is as follows:

<i>EcoRI</i>	3.0 kb	3.5 kb	
<i>HindII</i>	2.0 kb	4.5 kb	
<i>EcoRI</i> and <i>HindII</i>	2.0 kb	1.0 kb	3.5 kb

Construct a restriction map for the given data.

Solution:

If we consider all the three digestions, sum of length of fragments obtained from each digestion turns out to be 6.5 kb. Thus 6.5 kb is total length of the linear DNA used.

Since two fragments are formed in both *EcoRI* and *HindII* single digestions, each of the two restriction sites are present only once on the linear DNA.

Let the fragments in double digestion be

A=2.0 kb, B=1.0 kb, C=3.5 kb

then, in single digestion of *EcoRI*, fragments are

A+B=3.0 kb, C=3.5 kb

Enzymes used in Recombinant DNA Technology

and, in single digestion of *Hind*III , fragments are

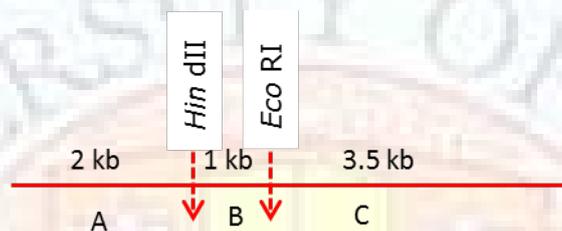
A=2.0 kb, B+C=4.5 kb

If we try to arrange the fragments then,

A+B

B+C

Therefore,



Example 2:

A circular DNA of 14 kb was subjected to single as well as double digestion with *Bam*HI and *Kpn*I enzymes. Data for fragments obtained for each digestion is as follows:

<i>B</i>	5.1 kb	5.4 kb	3.5 kb			
<i>K</i>	6.5 kb	1.8 kb	5.7 kb			
<i>B/K</i>	1.9 kb	4.6 kb	0.8 kb	1.0 kb	3.2 kb	2.5 kb

Construct a restriction map for the given data.

Solution:

If we consider all the three digestions, sum of lengths of fragments in each digestion turn out to be 14 kb. Thus 14 kb is total length of the plasmid DNA used.

Since three fragments each are formed in *Bam*HI and *Kpn*I single digestion, restriction sites for both the enzymes are present thrice on the circular DNA.

Let the fragments obtained from double digestion be

A=1.9 kb, B=4.6 kb, C=0.8 kb, D=1.0 kb, E=3.2 kb, F=2.5kb

then, in single digestion of *Bam*HI, fragments are

A+E=5.1 kb, B+C=5.4 kb, D+F=3.5 kb

Enzymes used in Recombinant DNA Technology

and, in single digestion of *Kpn* I, fragments are

A+B=6.5 kb, C+D=1.8 kb, E+F=5.7 kb

If we try to arrange these fragments then,

A+E

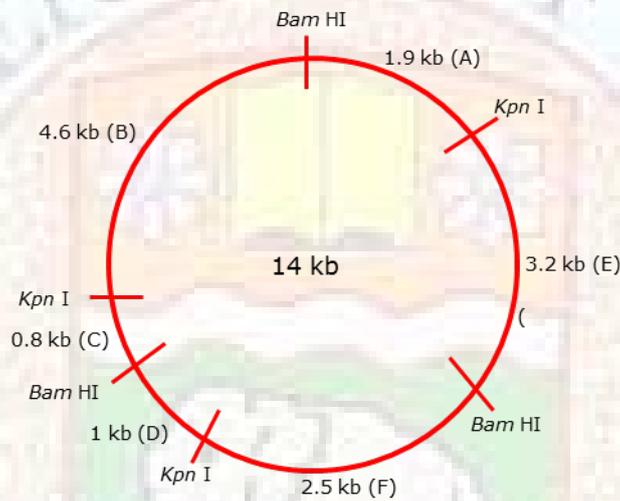
E+F

F+D

D+C

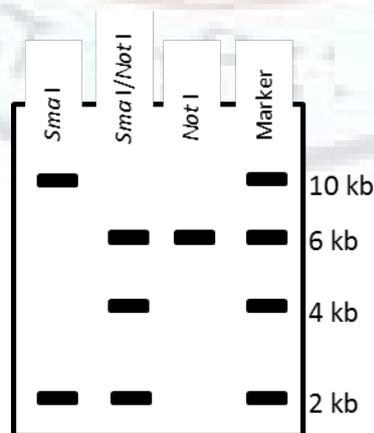
C+B

B+A



Example 3:

Following is the agarose gel profile of single and double digestion of a plasmid with *Sma*I and *Not*I. Find the number of sites for both *Sma*I and *Not*I. Draw the restriction map for the plasmid.



Enzymes used in Recombinant DNA Technology

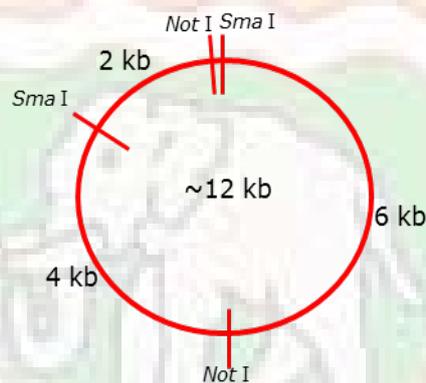
Solution:

Sum of fragment lengths in both *Sma*I single digestion and that of *Sma*I/*Not*I double digestion is 12 kb, but for *Not*I single digestion is 6 kb. This is not possible, because the total of all fragment sizes of all digestions should be equal. Therefore, it appears that there are two fragments of 6 kb each, formed by *Not*I digestion that migrate together on the agarose gel.

Thus, two fragments each are generated by *Sma*I and *Not*I single digestion respectively.

Also, the number of fragments obtained for double digestion are three instead of four as expected. Thus, probably the two restriction sites are too close to each other to form a visible band on gel.

Thus the restriction map of the above described plasmid would look as is shown in the figure.



A few video links are provided herein that will help the students to better understand restriction mapping.

<https://www.youtube.com/watch?v=8FqMUF96cPE>

<https://www.youtube.com/watch?v=kRUBRrd6Lyc>

The students can also go through the following link to find more numericals based on restriction mapping.

http://science.holeintheground.net/events/DesGene/restriction_mapping.pdf

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<http://m.learning.hccs.edu/faculty/tineke.berends/biol2416/genetics-biol-2416-lab-materials/restriction-mapping-of-mystery-dna/restriction-mapping-practice-problems/restriction-mapping-practice-problem>

Applications of restriction enzymes

Traditional Cloning

Traditional cloning is a robust methodology used in day to day experimentation in molecular biology laboratories. In this workflow, restriction endonucleases together with DNA ligases facilitate the formation of recombinant DNA. The procedure appears similar to the 'cut and paste' command in computer. The figure below shows the basic flowchart of how a 'DNA of interest' with flanking restriction sites can be isolated and be placed in a compatible vector with the same restriction sites. Once the plasmid vector is constructed with the required 'insert', it can be propagated in *E. coli*. Restriction enzymes are also used for post-cloning confirmatory tests that ensure if the insertions have taken place correctly.



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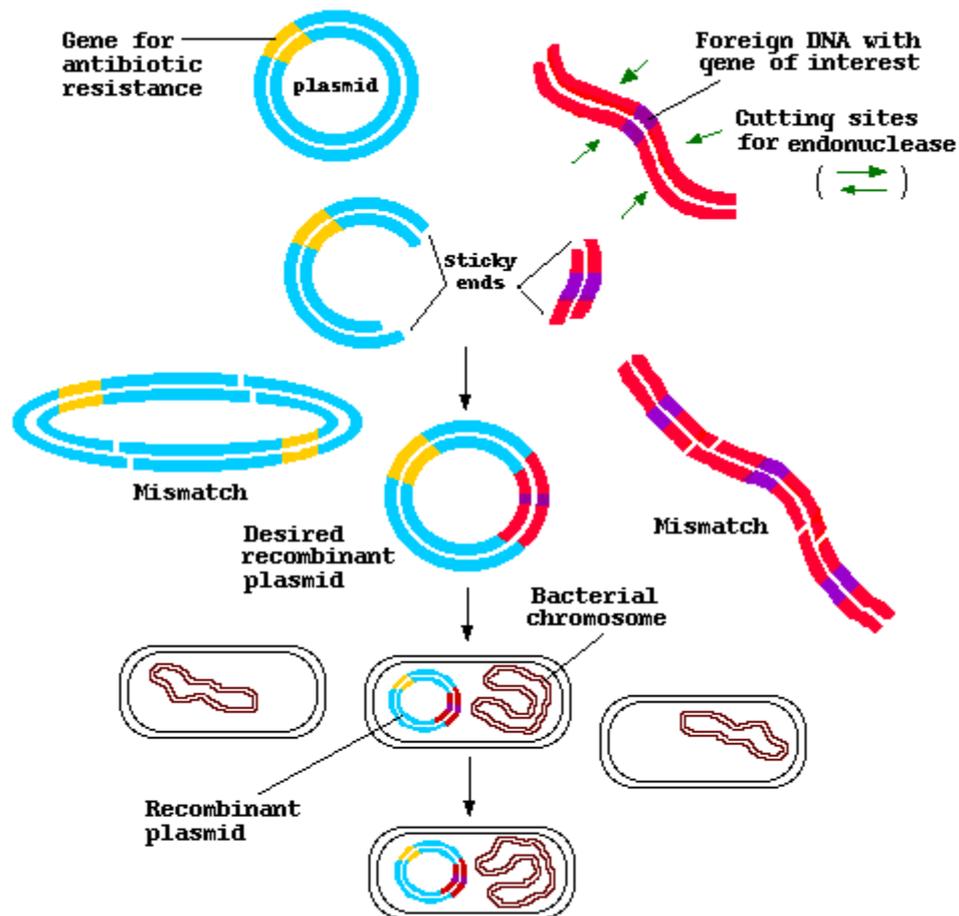


Figure:Diagrammatic depiction of workflow for traditional cloning. The chromosomal DNA is digested with suitable enzymes to release the DNA fragment of interest. The compatible plasmid vector is also digested with the same enzyme so that compatible ends are produced. With the help of DNA ligase the chromosomal fragment is inserted into the vector to construct the cloned vector. These procedures have enabled the cloning of recombinant DNA for their study and production of recombinant proteins.

Source: <http://whaleandwasp.wikispaces.com/Part+1-DNA+Cloning,+Recombinant+DNA,+Genetic+Engineering> (cc)

DNA Mapping

Enzymes used in Recombinant DNA Technology

Restriction enzymes can be used to construct a map of a given DNA that gives structural information about it. Daniel Nathans, in early 1970s, was the first to map the genome of virus SV40. Since then, DNA mapping is used to detect various DNA polymorphisms. DNA polymorphism refers to the differences in sequence between two DNA molecules. Usually two alleles of a gene are studied for any polymorphism between them. These polymorphisms include single nucleotide polymorphisms (SNP) and insertions/deletions (Indels). These can be detected by restriction mapping. If a restriction site has been changed due to a SNP or Indel, the number of fragments formed after the restriction digestion will be reduced. Likewise, if a new site has been created due to SNP or Indel, then additional bands will be seen on the agarose gel after electrophoresis. If some insertion or deletion occurs between two consecutive sites, it will result in increase or decrease of the fragment size. This kind of polymorphism that affects restriction fragment size is called as restriction fragment length polymorphism (RFLP).

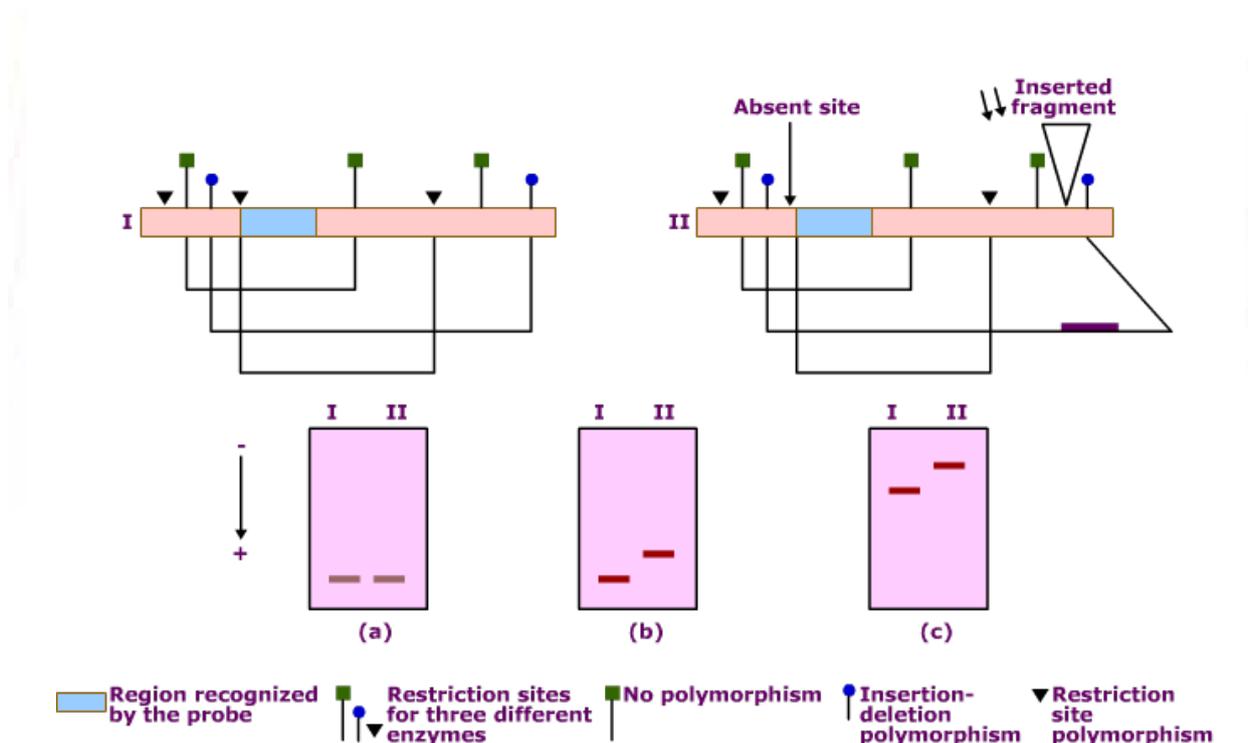


Figure: RFLP technique is used to detect polymorphism between two individuals, I and II. a. No polymorphism. b. Restriction site polymorphism. c. Insertion-deletion (Indel) polymorphism. Polymorphism between DNA from the two individuals digested using different enzymes can be viewed by gel electrophoresis.

Source: Ms. Manisha Sharma

Enzymes used in Recombinant DNA Technology

The above said DNA mapping techniques have many applications that include identifying genetic disorder loci, assessing the genetic diversity of populations and parental testing.

Understanding epigenetic modifications

Epigenetic modifications refer to various changes in the DNA that are heritable. These include methylation, acetylation, ubiquitylation, phosphorylation, histone modifications and various other modifications of the DNA molecules. It is known that methylated restriction sites are not acted upon by most of the restriction enzymes. Thus a change in the methylation status can be predicted by restriction maps. A DNA that has non-methylated restriction site will be cleaved by the enzyme and produce two fragments, whereas the one with methylated site will produce only one band when separated by agarose gel electrophoresis. Such studies can help in understanding the changes in the methylation patterns of the genome during the development of normal and stressed cells like cancer cells.

Summary

- Manipulation of DNA molecules at will is important for creating recombinant DNA.
- Various enzymes categorized as nucleases, ligases, polymerases and other DNA modifying enzymes are used in molecular cloning for DNA manipulations.
- Nucleases are enzymes that cleave the phosphodiester bond between the nucleotides in a nucleic acid molecule.
- Exonucleases cleave the terminal phosphodiester bond, whereas endonucleases act upon the internal phosphodiester bonds
- Exonucleases can be categorized on the basis of whether they cut one strand (exonuclease III) or both the strands (Bal31) of DNA molecules.
- Endonucleases can be categorized into three major categories. S1 nuclease acts on single stranded DNA whereas DNase I acts on both double and single stranded DNA. Restriction endonucleases identify specific sites that are precisely cleaved by them.
- DNA Ligases join the 5' phosphate and 3' hydroxyl to form the phosphodiester bond. It can achieve both blunt end and sticky end ligation.

Enzymes used in Recombinant DNA Technology

- DNA polymerases are enzymes that enable new strand synthesis onto a pre-existing strand.
- *E. coli* polymerase I enzyme has both polymerase as well as bidirectional exonuclease activity. It can be cleaved to produce a fragment called Klenow fragment that has only polymerase and 3'-5' exonuclease activity.
- A DNA polymerase isolated from bacterium *Thermus aquaticus* is resistant to heat denaturation. This enzyme finds major applications in amplification of DNA by the technique called PCR.
- Reverse transcriptase is a polymerase that uses RNA template for synthesizing complementary DNA strand. It is obtained from retroviruses.
- Alkaline phosphatase removes 5' phosphate group from the DNA molecule.
- Polynucleotide kinase transfers the phosphate group to the 5' end of DNA strand.
- Terminal transferase transfers the dNTPs to the 3' end of DNA.
- Restriction endonucleases were discovered by Nathan, Arber and Smith in the year 1978 and received Nobel Prize for it.
- In bacteria, restriction enzymes restrict the invading viral DNA by cleavage, whereas its own DNA is protected from cleavage as result of modification (methylation) of nucleotides in the recognition site. This phenomenon is called as 'restriction-modification' system.
- Restriction endonucleases are of three types. Type I and type III are large complex enzymes with complex recognition sites. They cleave the DNA in a non-specific manner.
- Type II restriction endonucleases are comparatively smaller enzymes that bind to DNA as homodimers and cut the DNA precisely within or near the recognition site.
- The recognition sequences for type II restriction enzymes are symmetric and generally 4 to 8 bp long.
- Restriction enzymes either produce blunt or sticky ends.
- Different restriction enzymes with same recognition sequences are called as isoschizomers.
- Restriction mapping is a method of determining the relative positions of restriction sites on a piece of circular or linear DNA. This involves a series of single and double digestions and separating the fragments on an agarose gel electrophoresis.
- Various applications of restriction endonucleases include traditional cloning, DNA mapping and understanding epigenetic modifications.

Enzymes used in Recombinant DNA Technology

Exercise

Essay Type Questions

1. Why discovery of restriction endonucleases is considered as a landmark in the field of recombinant DNA technology?
2. Distinguish between the catalytic activities of alkaline phosphatase and polynucleotide kinase. Discuss the applications of the two enzymes.
3. Elaborate various characteristic features of recognition sites of type II restriction endonucleases.

Short notes

1. Klenow Fragment
2. S1 Nuclease
3. Role of restriction enzymes in bacteria
4. Applications of restriction enzymes
5. Reverse Transcriptase
6. Palindromic sequences

Expand the following

1. RFLP
2. INDEL
3. SNP
4. PCR
5. RT

Glossary

Bacteriophage: They are bacteria infecting viruses.

DNA ladder: A mixture of DNA fragments, used in gel electrophoresis as size markers.

DNA ligase: An enzyme that, in the cell, repairs single-stranded discontinuities in double-stranded DNA molecules. Purified DNA ligase is used in gene cloning to join DNA molecules together.

Enzymes used in Recombinant DNA Technology

DNA polymerase: An enzyme that synthesizes DNA on a DNA or RNA template.

Double digestion: Cleavage of a DNA molecule with two different restriction endonucleases simultaneously.

DNA sequencing: Determination of the order of nucleotides in a DNA molecule.

Endonuclease: An enzyme that breaks phosphodiester bonds within a nucleic acid molecule.

Exonuclease: An enzyme that removes nucleotides from the ends of a nucleic acid molecule.

Gel electrophoresis: Electrophoresis performed in a gel matrix so that molecules of similar electric charge can be separated on the basis of size.

Homopolymer tailing: Attachment of a sequence of identical nucleotides (e.g., CCCC) to the end of a nucleic acid molecule. It is generally done by terminal transferase.

Host-controlled restriction: A mechanism by which some bacteria prevent phage attack through the synthesis of a restriction endonuclease that cleaves the non-bacterial DNA.

Indel: A position where a DNA sequence has been inserted into or deleted from a genome.

Insert: DNA fragment that is inserted in the cloning vector

Klenow fragment (of DNA polymerase I): A DNA polymerase enzyme, obtained by proteolysis of *E. coli* DNA polymerase I, used mostly in chain termination DNA sequencing.

Methyl Transferase: An enzyme that adds methyl group to the nucleotides in a DNA molecule.

Nick: A single-strand break, involving the absence of one or more nucleotides, in a double-stranded DNA molecule.

PCR (Polymerase Chain Reaction): A technique that enables amplification of a DNA molecule using thermostable polymerases.

Enzymes used in Recombinant DNA Technology

Plasmid: A usually circular piece of DNA, primarily independent of the host chromosome, often found in bacteria and some other types of cells. It is generally used in recombinant DNA technology as cloning vector.

Polylinker: A synthetic double-stranded piece of DNA carrying a number of restriction sites.

Primer: A short single-stranded oligonucleotide that attaches to the template strand by base pairing and initiates the synthesis of the DNA strand by a DNA polymerase enzyme.

Recombinant DNA: A DNA molecule created in the test tube by ligating together pieces of DNA that are not normally contiguous.

Recombinant DNA technology: All of the techniques involved in the construction, study and use of recombinant DNA molecules.

Restriction endonuclease: An endonuclease that cuts DNA molecules only at specific nucleotide sequences.

Restriction fragment length polymorphism (RFLP): A mutation that results in alteration of a restriction site and hence a change in the pattern of fragments obtained when a DNA molecule is cut with a restriction endonuclease.

Restriction Map: A map showing the positions of different restriction sites in a DNA molecule.

Retrovirus: A virus with an RNA genome.

Reverse transcriptase: An RNA-dependent DNA polymerase that uses RNA template to synthesize a complementary DNA molecule.

Taq DNA polymerase: The thermostable DNA polymerase used in PCR, isolated from *Thermus aquaticus*.

Template: A single-stranded polynucleotide (or region of a polynucleotide) that directs synthesis of a complementary polynucleotide.

Vector: A DNA molecule, capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule.

Enzymes used in Recombinant DNA Technology

References

- Brown, T.A., Gene cloning and DNA analysis – an introduction, sixth edition, John Wiley & Sons Ltd. Publication, West Sussex, 2010.
- Green, M.R. and Sambrook, J., Molecular cloning – a laboratory manual, fourth edition, Cold spring harbour laboratory press, New York, 2012.

