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SYNTHETIC GENE NETWORKS

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15.1 INTRODUCTION

Advances in molecular manipulation techniques, together with an ever-increasing accumulation of genetic information, are progressively opening new possibilities for gene therapy and biomedical engineering. By combining naturally occurring genetic components in unique ways, it has become possible to artificially engineer genetic networks that possess increasingly sophisticated functional capabilities. By analogy to electronic circuit engineering, the desired characteristics of such networks can be rationally designed and tested through predictive modeling. Similarly to electrical networks, genetic networks also possess ''input'' and ''output'' functionality such that they are capable of monitoring and responding in highly defined mechanisms. The creation of synthetic networks from well-defined modular components has enabled researchers to investigate and test many network characteristics found in natural genetic networks. It is from an applied perspective, however, that synthetic genetic networks represent a truly exciting innovation. It is not difficult to envisage applications where synthetic networks could be used to manipulate cellular behavior in a highly orchestrated way. While these concepts are still in their infancy, significant progress has been made in the creation of first-generation synthetic networks, which will one day enable the engineered control of cellular function to become a viable reality.

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This chapter begins by describing the modular genetic components that form the building blocks of engineered genetic networks. It then describes the development of both simple and complex networks, many of which were initially developed in prokaryotic systems, but which have been subsequently extended to eukaryotic systems. The focus is upon describing networks that have been experimentally tested and validated. It does not cover the extensive modeling and computational work that has been conducted on either synthetic or natural genetic regulatory networks (readers are referred to Chapter 7). Advances in network functionality have been made on both the input and output dimensions. Examples of output functionality include the generation of stable behavior, such as bistable toggle and hysteric switches, and dynamic behavior such as an oscillatory network. From an input perspective developments include the creation of logical information ''gates,'' where a range of input combinations produce highly defined outputs in a manner directly analogous to electrical circuits; the development of transcriptional cascades, which have enabled the range of inputs to a network to be greatly increased; and the development of novel sensory networks which, for example, can detect inputs within a defined concentration range, or respond precisely to a rising level of an input. The chapter concludes by presenting the initial first steps into the emerging field of semisynthetic networks. These are prosthetic genetic networks that are capable of responding to physiological cues so that they are effectively integrated into the host-cell's biology. Such networks, in response to acute or pathological cues, hold great promise for the controlled manipulation of cellular processes such as protein synthesis, metabolism, cell growth, and differentiation.

15.2 NETWORK BUILDING BLOCKS

While synthetic in the sense that they are artificially designed and created, synthetic genetic networks are actually engineered from naturally occurring genetic components. A discussion of these networks requires a basic understanding of these components and the manner in which they interact. While gene expression can be regulated and artificially manipulated at a number of levels, the networks described below have only utilized a limited number of transcriptional control elements. Hence, this overview is limited to the mechanisms and components that have been used in these systems. A comprehensive overview of other gene control systems and their application can be found in several recent reviews [1–3].

Transcriptional control operates at the level of mRNA synthesis through the use of inducible transcriptional activators and repressors that are capable of binding naturally occurring or specifically engineered promoters. The majority of systems utilize bacterial response regulators or activators that, upon binding to a target promoter, inhibit or activate transcription respectively. Binding of a specific molecule to the response regulator induces an allosteric change leading to disassociation of the regulator from its cognate promoter.

Prokaryotic gene control systems generally use inducible repressors and activators drawn from well-documented genetic operons such as the *lac* operon of *Escherichia*

coli [4], the tetracycline-resistance transposon Tn*10* [5], or the λcI repressor of bacteriophage lambda [6]. In each case, the respective response regulator binds to a DNA sequence, typically a short tandem repeat referred to as the "operator," located within or adjacent to a promoter where it either enhances transcription or sterically hinders the initiation of transcription. By substituting operators across different strength promoters it has been possible to generate inducible systems with varied induction characteristics [7].

Bacterial response regulators also form the basis of synthetic eukaryotic gene regulation systems although given transcriptional differences they require adaptation. This has been successfully achieved for many bacterial response regulators by placing the operator for the response regulator adjacent to an eukaryotic compatible promoter [8]. The response regulator thus acts as a heterologous DNA-binding protein (DBP) whose association with the desired promoter can be controlled through addition of an appropriate inducer. If the operator is placed close to an strong constitutive promoter (e.g., P_{CMV}, cytomegalovirus immediate early promoter), DBP binding can sterically prevent the initiation of transcription by RNA polymerase II machinery. Alternatively, transcription can be actively repressed by fusing a eukaryotic transcriptional silencer, such as the Kruppel-associated box protein (KRAB), to the DBP [9]. Such systems are referred to as ON-type systems, as the addition of an inducer leads to derepression of transcription (Fig. 15-1). In an OFF-type configuration, in which addition of inducer leads to transcriptional silencing, a transcriptional activation domain, such as the *Herpes simplex* virus VP16, is fused to the DBP [10]. By placing the corresponding operator site adjacent to a minimal promoter (e.g., $P_{hCMVmin}$, minimal version of the human cytomegalovirus immediate early promoter), DBP binding activates transcription from an otherwise silent minimal promoter. Addition of an inducer results in subsequent deactivation of transcription.

As many prokaryotic antibiotic response regulators have been well described, and given the low interference of many antibiotics with eukaryotic biology, they represent an ideal class of inducible DBPs for eukaryotic gene control. Using the aforementioned configurations, eukaryotic gene control systems responsive to tetracyclines [11], streptogramins [12], and macrolides [13] amongst others have been developed. As these gene control systems do not interfere with each other, they can be readily combined. For this reason, and their nonpleiotrophic effects, they have formed the basis of most eukaryotic synthetic gene networks. A list of the common transcriptional control elements used in the assembly of both prokaryotic and eukaryotic synthetic gene networks is provided in Table 15-1.

15.3 CHARACTERIZATION OF SIMPLE AND COMPLEX NETWORKS

The past decade has seen a progressive increase in the development and application of both prokaryotic and eukaryotic synthetic networks. In some cases, these networks have been relatively simple and have been used to test and investigate naturally occurring phenomena. In other cases, the networks exhibit far greater complexity as they seek to reproduce or create much more sophisticated functionality. When adopting

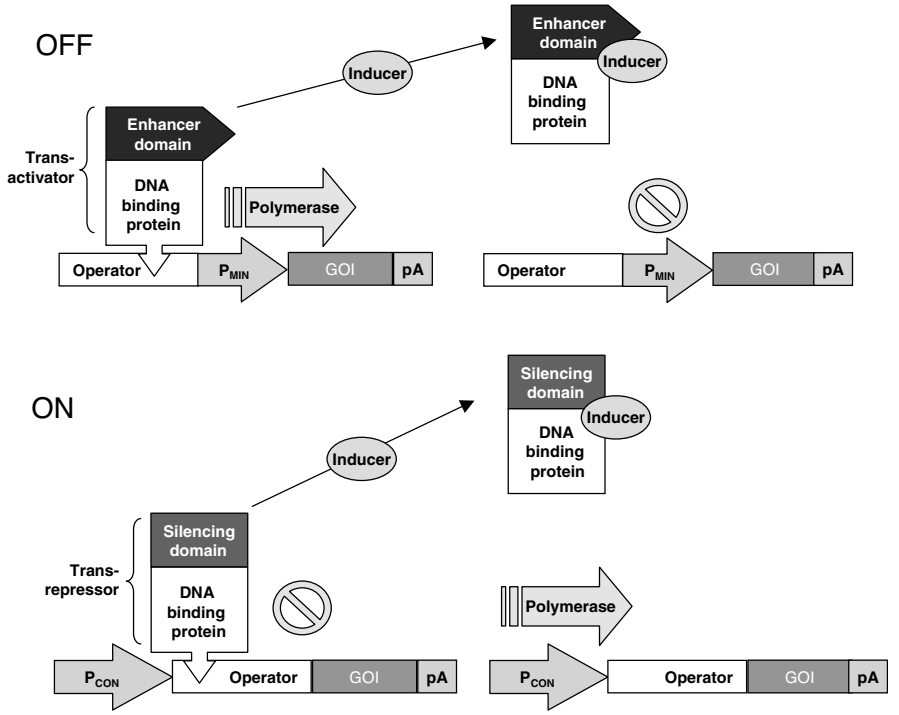

Figure 15-1 Molecular configuration of OFF and ON synthetic eukaryotic gene regulation. In the OFF configuration, a DNA-binding protein—typically a bacterial transcriptional repressor—binds a specific operator site placed adjacent to a minimal promoter (P_{MIN}). An activation domain fused to the DBP activates polymerase-mediated transcription of a gene of interest (GOI). Addition of an inducer specific to the DBP causes an allosteric change resulting in disassociation of the transactivator with subsequent transcriptional arrest. In the ON configuration, the DBP is fused to a repressor domain. Binding of DBP-TR to an operator site placed adjacent to a constitutive promoter (P_{CON}) represses transcription of the GOI. Again, addition of a DBP specific inducer results in transrepressor disassociation although in this configuration, repression is abolished resulting in expression of the GOI [8].

the electrical circuit analogy it is possible to describe synthetic genetic networks in terms of their input functionality—how the network receives and integrates specific signals as well as their output functionality—how the network produces and maintains a specific pattern of expression. Given that much of the pioneering work in synthetic circuits was directed toward producing novel patterns of gene expression, it is expedient to commence with network descriptions of output functionality.

In considering the design of a synthetic genetic network for a biological application it is useful to imagine what kind of functions one might wish to create. Thus, some applications may benefit from a mechanism that ensures a network produces a consistent and stable response even when there are considerable random fluctuations in either network components, inducer concentrations, or cellular components more broadly. For other applications, one may require a system that produces more than one

Table 15-1 Common genetic transcriptional components used in the reaction of synthetic genetic networks

DNA-Binding Protein	System Application	Engineered Regulatory Protein	Inducer	Response to Inducer	References
TetR	Prokaryotic	—	Doxycycline, aTc	Derepression	[5]
LacI	Prokaryotic	—	IPTG	Derepression	[4]
λcI	Prokaryotic	—	Temperature	Derepression	[6]
NRI	Prokaryotic	—	Phosphorylation	Activation	[94]
LuxR	Prokaryotic	—	Acyl-homoserine lactone	Activation	[95]
LacI	Eukaryotic	—	IPTG	Derepression	[96]
TetR	Eukaryotic	TetR-VP16 (tTA)	Tetracycline,	Deactivation	[11]
rTetR	Eukaryotic	rTetR-VP16	Doxycycline, aTc	Activation	[11]
Pip	Eukaryotic	Pip-KRAB	Streptogramins	Derepression	[12]
Pip	Eukaryotic	Pip-VP16	Streptogramins	Deactivation	[12]
E	Eukaryotic	E-KRAB	Macrolides	Derepression	[13]
E	Eukaryotic	E-VP16	Macrolides	Deactivation	[13]
ScbR	Eukaryotic	ScbR-VP16	Butyrolactones	Deactivation	[97]
Gal4	Eukaryotic	Gal4-VP16	Mifepristone	Deactivation	[56]
HIF-1α	Eukaryotic	–	Hypoxia	Activation	[98]

aTc, anhydrotetracycline; IPTG, isopropyl-β-D-thiogalactopyranoside; KRAB, Kruppel-associated box protein-derived transrepressor domain; VP16, *Herpes simplex* viral protein 16-derived transactivation domain.

discrete expression state. A mechanism that "remembers" what conditions the network has been exposed to may be useful in applications where only a transient pulse of an inducer is required or expected. A mechanism that not only remembers the past but also reacts differently to subsequent changes would also be desirable. Finally, a mechanism that produces continuous oscillations in expression readout may be highly practical where repeated temporal expression is required. All of these mechanisms have their counterpart in natural biological systems where they represent the molecular controls for numerous basic cellular functions ranging from cellular differentiation, cell-cycle control, and circadian rhythms. It is therefore not surprising that genetic engineers have applied considerable effort to synthetically reproduce these mechanisms. Apart from being useful tools, such synthetic networks also shed considerable light on how the equivalent mechanism occurs in a natural system.

15.3.1 Expression Stability

To produce a unified and consistent outcome a biological process, whether it involves metabolic homeostasis or cellular growth and development, must be capable of withstanding a certain degree of variation and difference [14–16]. As cellular biochemical networks are highly interconnected, a perturbation in reaction rates or molecular concentrations may affect multiple cellular processes including transcription, translation, and RNA and protein degradation—all of which impact gene expression. Systems that, despite the influence of considerable variation and random perturbation, are capable of remaining close to a steady state can be characterized as stable (or robust). Existing artificial gene regulation systems are typically highly susceptible to even modest fluctuations in regulatory components, which can significantly affect expression performance. In contrast, many natural gene networks intrinsically exhibit high stability. A natural question, therefore, is which mechanism(s) would enable a network to withstand such variation? A key development in our understanding of how stability is maintained was through the discovery of autoregulatory feedback loops in which proteins, directly or indirectly, influence their own production [17]. An autofeedback mechanism can either be negative, in which a protein inhibits its own production, or positive, in which a protein stimulates its own production.

Although it had been proposed that autoregulatory negative feedback loops provide stability, thereby limiting the range over which the concentrations of network components fluctuate, it was Becskei and Serrano who first demonstrated how a negative feedback mechanism can increase expression stability (Fig. 15-2) [18]. By fusing green fluorescent protein (GFP) to the tetracycline-responsive repressor protein (TetR) they were able to measure variations in TetR expression (measured by coefficient of variation in fluorescence intensity) across a population of *E. coli*. In using an established prokaryotic gene regulation system they created a negatively autoregulated system in which TetR inhibits its own transcription, as well as an unregulated system where TetR has no influence upon its transcription rate. Consistent with predictions from mathematical modeling, the experimental data showed that the autoregulated system exhibited a threefold narrower variation in expression levels

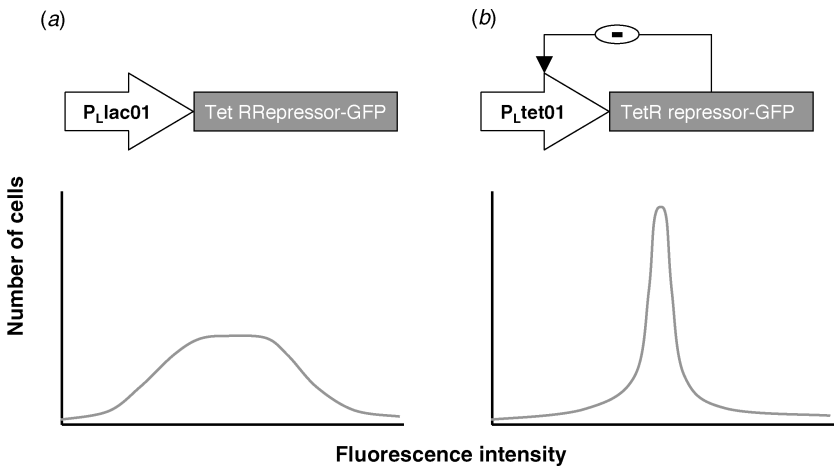

Figure 15-2 Expression profile of (a) an unregulated genetic system compared to (b) an equivalent system utilizing negative autofeedback. Both systems were based on the same architecture in which a promoter was used to control expression of a fusion protein consisting of the tetracycline repressor (TetR) and GFP in *E. coli*. In the regulated system, the promoter contained two tetracycline repressor operator modules (P_{Ltet01}). Negative feedback occurs as TetR repressors transcription from P_{Ltet01}. In the unregulated system, TetR was prevented from interacting with the promoter by substituting the TetR operator with a different (LacR) operator (or by the functionally equivalent step of mutating the TetR-DNA-binding domain). In this way, the feedback mechanism was eliminated without altering other aspects of the genetic system. The resulting distribution of expression states for the unregulated system was wider than the corresponding distribution for the negative feedback system thus demonstrating the higher stability of a genetic system employing autofeedback [18].

than the unregulated system. Furthermore, through the addition of anhydrotetracycline (aTc), which causes TetR to dissociate from its cognate operator thereby reducing feedback repression, it was possible to introduce variation levels into the autoregulated system which approached the variation levels observed in the unregulated system. Hence, in this simple synthetic network negative feedback provides a mechanism for ensuring a more stable expression state. This is consistent with observations of expression stability in natural systems for either prokaryotes or eukaryotes in which transcription factors are known to use both positive and negative autoregulation to control their own production [19,20].

A key requirement for many networks and biological functions is the capacity to produce more than one discrete stable expression state. The creation of binary, or even multiple, expression states raises a number of possibilities for how a network can transition from one state to the other (Fig. 15-3). In a classic graded expression system, an increase in the concentration of an inducer generates a graded (or continuous) transcriptional response that, in a graphical representation, resembles a sigmoid shape. This pattern is due to transcriptional cooperativity in which initial binding of a transcriptional regulator to a promoter enhances subsequent binding of further regulators to the same promoter. This can either be due to cooperative binding or regulator multimerization [21]. Yet, in some systems the switch from one state to

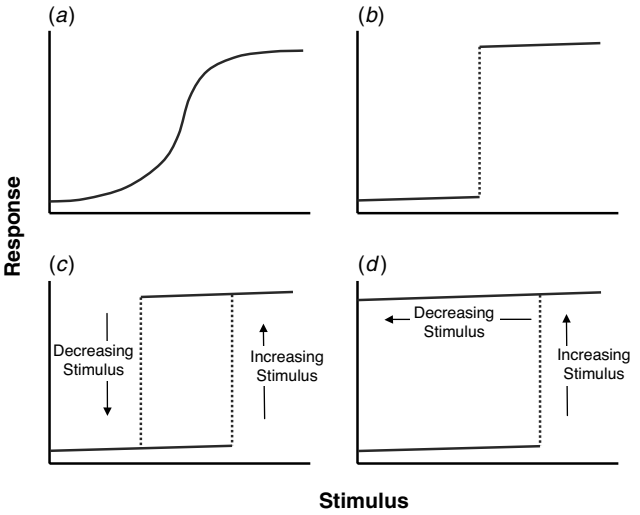

Figure 15-3 Stimulus–response profiles for (a) graded, (b) generic bistable, (c) hysteretic bistable, and (d) self-sustaining bistable genetic networks.(a) In a graded genetic system an increasing stimuli is progressively converted into an increasing response, which often adopts a sigmoidal pattern due to activator or repressor cooperativity. (b) In a generic bistable network the system exhibits quasi-discontinuous behavior whereby it only resides in one of two alternative steady states and not an intermediate state. Through changes in stimuli beyond a threshold point it is possible to switch or "toggle" the system from one state to another. (c) A hysteretic bistable network requires differing threshold stimuli levels to switch between steady states depending upon the starting state of the system. (d) In a self-sustaining bistable network the system remains in one steady state indefinitely even after the stimulus used to create that state has been removed [37,38].

another can be so swift as to almost represent discontinuous behavior. With increasing sophistication such a quasi-discontinuous switch can have different switching dynamics depending upon its starting point (i.e., hysteresis) or may even be self-sustaining (i.e., toggle) and/or irreversible. In addition to providing a means of achieving a single stable expression level, feedback regulation is also an important mechanism for producing a binary, or bistable, expression state in response to different input parameters [22].

15.3.2 Binary Expression

A graded transcriptional response typically results in a unimodal expression pattern where, when viewed across a cell population, there is no evident separation of expression states (Fig. 15-4). This remains so even when an inducer is used to increase expression—the resulting distribution is simply shifted upward reflecting an overall increase in expression across the entire cell population. Using a common tetracycline-responsive transactivator (TetR-VP16) and GFP reporter, it has been demonstrated that a simple autofeedback mechanism can create a binary expression readout in *Saccharomyces cerevisiae* [22]. By introducing positive feedback into the classical

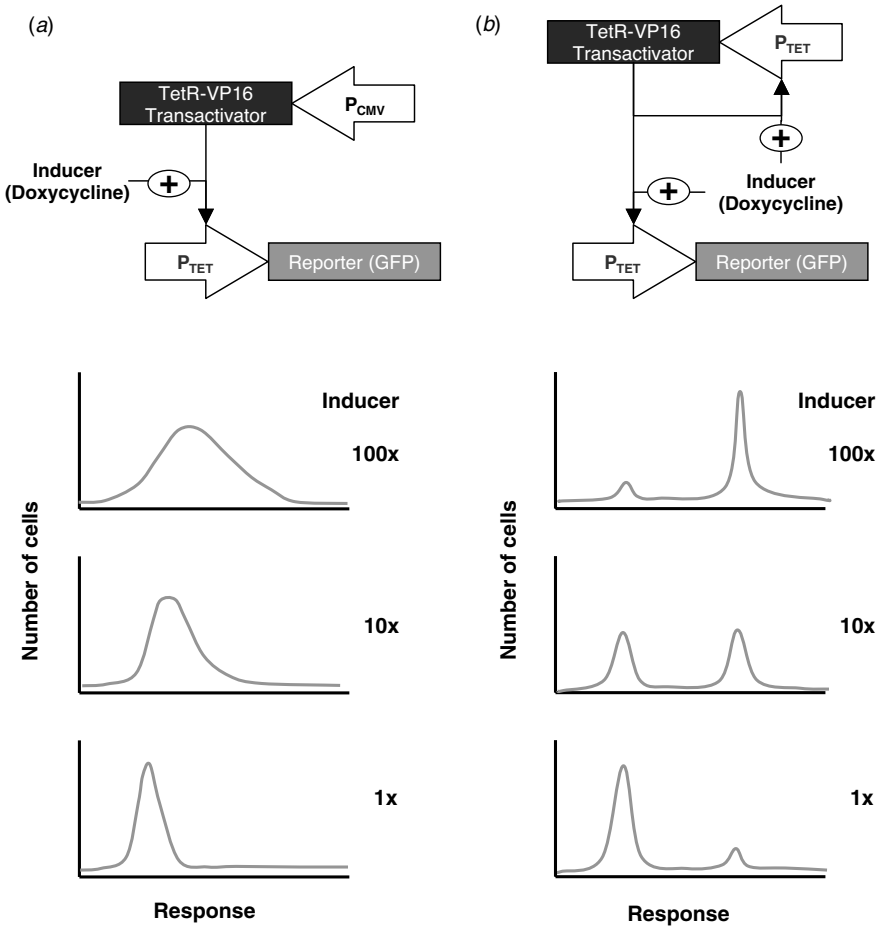

Figure 15-4 Graded response profile of (a) a classic transcription control system relative to (b) a bistable expression profile using a positive autofeedback mechanism. Both the classic and autoregulated systems were based on the tetracycline-dependent transactivator (TetR-VP16) eukaryotic transcriptional system. In the classic graded system, a strong constitutive CMV promoter (P_{CMV}, cytomegalovirus immediate early promoter) was used to transcribe TetR. For the autoregulated system, the constitutive CMV promoter was replaced with a TetR-inducible promoter (P_{TET}) thereby creating a positive autofeedback loop. In both cases, a chromosomally integrated TetR-inducible GFP reporter construct was used to assess expression profiles. The classic graded system exhibits a unimodal distribution pattern which, following addition of doxycycline, shifts progressively to the right. The autoregulated system exhibits a bimodal distribution pattern that does not shift upon inducer addition. Rather, doxycycline addition concomitantly alters the proportion of cells residing in either of the two expression states [22,38].

TetR-VP16 transcription system, in which expression of TetR-VP16 positively influences its own production rate, a binary distribution pattern was produced whereby the cell population was clearly divided into discrete pools of ON and OFF cells. Importantly, following progressive administration of increasing inducer levels, the

pools did not significantly shift relative to each other, but rather the distribution of cells between the ON and OFF pools changed inversely. This indicates that the autofeedback mechanism prevents cells from adopting an intermediary expression status such that they can only reside in one of the two possible states. Despite, the delineation of expression into one of two states, it was observed that individual cells did not necessarily remain in a fixed state. Across a range of inducer concentrations, a certain proportion of cells randomly flipped between states indicating that the binary states were not entirely stable.

15.3.3 Bistability

A binary expression system that does not exhibit random switching between two expression states is said to be bistable. Bistability is a minimal requirement for a network to possess memory in which the state of the network stores information about its past [23]. In addition to bistability, a network can only possess memory where it remains in an expression state long after the stimulus used to force it into that state has been removed. Such a self-sustaining mechanism is analogous to a typical light switch or toggle. Switching a light ON or OFF only requires a single transient, rather than a persistent, input.

15.3.3.1 Bacterial Toggle A pioneering step in the development of synthetic networks was the creation of a plasmid-based bistable expression switch in *E. coli* [24]. The switch was constructed from two inducible bacterial repressors, transcribed from two similar strength promoters selected such that each repressor inhibited the promoter of the opposing repressor (Fig. 15-5). By placing a fluorescent reporter gene (GFP) downstream of one of the repressors it was possible to monitor which repressor was currently active, and thereby the expression status of the network. Owing to the mutually inhibitory arrangement of the two repressor genes, the network was capable of one of the two binary states: A HIGH state in which the first repressor and the downstream GFP reporter are transcribed from the second promoter, and a LOW state in which the second repressor is transcribed from the first promoter. In the absence of relevant inducers, the network can initially adopt either state, but once committed remains in the adopted state indefinitely. However, through the addition of a relevant inducer, it was possible to switch the network from one state to the other. The addition of an inducer to the active repressor enables the opposing repressor to be maximally transcribed. Once the opposing repressor has reached a certain level it represses transcription of the initially active repressor. As the prevalence of the opposing repressor over the initially active repressor becomes self-perpetuating, the inducer can be withdrawn and the network continues indefinitely in its altered state. In this manner, the network behaves as a bistable "toggle" switch in which the maintenance of either expression state does not require an ongoing inducer or stimulus. Furthermore, the status of the toggle could be maintained across cell generations indicating that network memory could be passed to progeny cells.

Six different toggle switches, employing different promoter-repressor pairs, were designed and characterized. Together with a mathematical approach it was possible

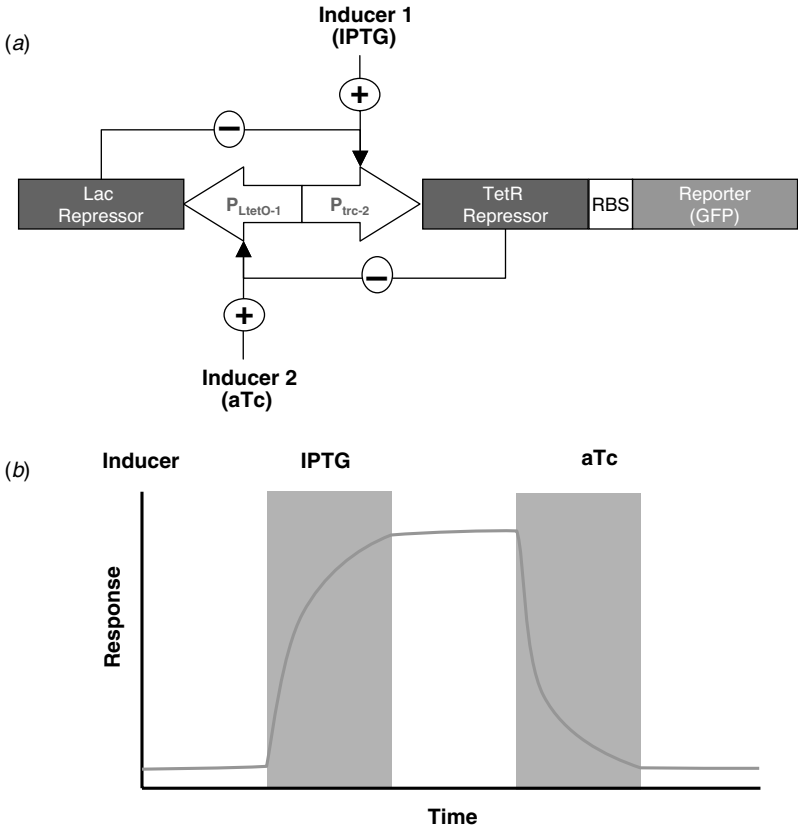

Figure 15-5 Engineered self-sustaining bistable "toggle" switch in *E. coli*. (a) Genetic design and (b) response profile.The genetic toggle switch was constructed from two sets of mutually opposing repressors/promoters. In the depicted configuration, the TetR repressor inhibits transcription of the Lac repressor from the $P_{LtetO-1}$ promoter. The Lac repressor in turn inhibits transcription from a P_{trc-2} promoter of the TetR repressor, a downstream ribosome-binding site (RBS) and a reporter gene (GFP). In the absence of inducers, both repressors mutually inhibit each other resulting in a low expression state. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) results in derepression of the Lac repressor and subsequent full expression of the TetR repressor and GFP (a HIGH expression state). Conversely, addition of aTc causes deinhibition of the TetR repressor with subsequent full expression of the Lac repressor (a LOW expression state). In both cases, only a transient pulse of inducer is required to enable the opposing repressor to be maximally transcribed until, in a self-perpetuating manner, it stably represses the originally active promoter [24].

to predict and assess many of the properties required for bistable switching. The interaction between toggle components was described using a simple differential equation model based upon rate equations for each repressor's production, repression activity, and degradation/dilution. Importantly, two criteria were found to be critical for robust bistability. First, each repressor had to be capable of cooperative repression at the promoter to which it binds. Mathematical modeling predicted that it is not the

strength of the promoters *per se*, but rather the degree of cooperative repression that has a direct impact upon system robustness, defined as the ability to avoid stochastic switching between expression states. Thus, even weak promoters should be capable of bistability as long as cooperative repression is sufficiently high. Second, it was predicted that the rates of synthesis of the two repressors must be evenly balanced. This was empirically confirmed in one set of toggle components that were only capable of a single steady state due to uneven repressor synthesis rates. The different toggles also provided insight into the dynamics of switching time—defined as the time required for the relevant inducer to mediate a sustainable switch—although in this case the primary determinant was surprisingly not the rate of elimination of the initially active repressor protein. In one toggle system, requiring IPTG-induced inhibition of a repressor, the switching time was 6 h. In contrast, when a temperature sensitive repressor was employed, the immediate inhibition of the repressor caused by thermal destabilization resulted in sustainable switching occurring within 35 min.

The construction and characterization of several toggle switches illustrates the increasing utility of synthetic genetic networks. The construction of synthetic networks with varying properties enabled the testing and empirical validation of physical and mathematical approaches to gene regulation. While these approaches have been previously applied it has not been possible to test their predictions. Synthetic gene networks are a useful tool for this purpose and should permit the qualitative behavior of gene regulation to be studied and described in a manner analogous to that already conducted for enzyme regulation. It also highlights the importance of correct component selection and compatibility in creating a network with desired specific behavior [25].

15.3.3.2 Mammalian Toggle A synthetic mammalian toggle switch capable of bistable expression has also been created, employing the same network architecture used in the synthetic *E. coli* toggle switch [26]. In this case, however, two eukaryotic transrepressor control systems were used: the E-KRAB system that is responsive to macrolide antibiotics such as erythromycin (EM) and the Pip-KRAB system responsive to streptogramin antibiotics such as pristinamycin (PI) (Fig. 15-6). A mutually opposing configuration, whereby each system represses expression of the other systems' transrepressor, generated two alternate stable expression states.

In the absence of either inducer molecule the network is balanced so long as both systems exhibit the same (low) expression levels with neither expression system able to prevail over the other. However, this balance can be tipped by addition of either inducer molecule in which case expression from one system is increased while expression from the other system is simultaneously repressed. Depending on the inducer added, the result is one of the two alternate expression states in which one transrepressor is expressed much more highly than the other. By placing a reporter gene (i.e., SEAP) immediately downstream of one of the transrepressors (i.e., Pip-KRAB), it was possible to tie SEAP expression to Pip-KRAB expression thereby obtaining a readout of the network status. A HIGH response, corresponding to high (or derepressed) Pip-KRAB expression, was obtained following induction with erythromycin whereas a LOW response, corresponding to low (increased repression)

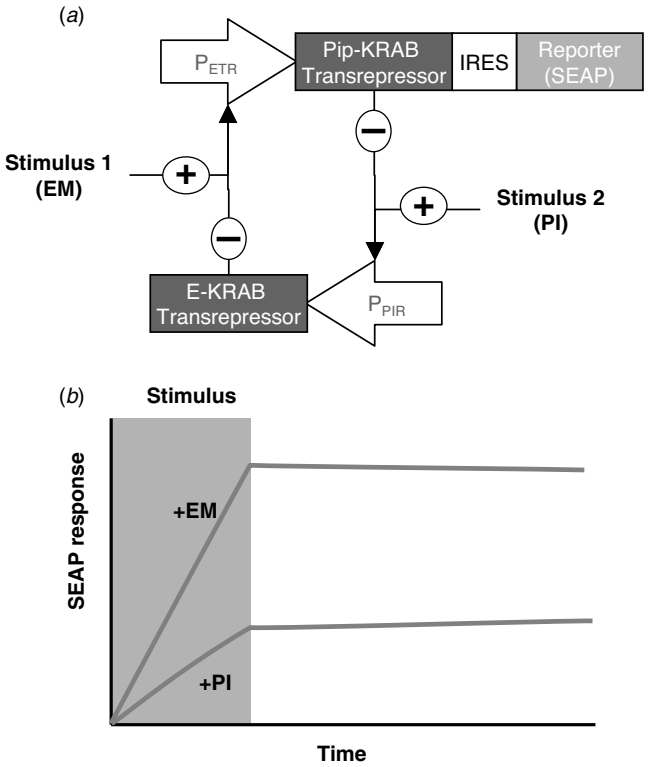

Figure 15-6 (a) Genetic construction and (b) response profile of engineered self-sustaining "toggle" switch.The mammalian toggle switch was assembled using two antibiotic-inducible transrepressor control systems, which were arranged to repress each other's expression. Erythromycin-inducible E-KRAB repressors transcription of Pip-KRAB and the human model reporter protein SEAP (human placental secreted alkaline phosphatase)—whose translation is modulated by an internal ribosome entry site (IRES). Pristinamycin I-inducible Pip-KRAB in turn repressors expression of E-KRAB. In both cases, addition of the respective inducer inhibits the repressive effect of the responsive transrepressor. Transient administration of EM results in P_{ETR}-driven coexpression of Pip-KRAB and SEAP with concomitant repression of E-KRAB (a HIGH response), whereas transient administration of PI results in P_{PIR} driven expression of E-KRAB with concomitant corepression of Pip-KRAB and SEAP (a LOW response). Both responses were maintained in a steady state following removal of relevant inducer molecules (nonshaded region) [26].

Pip-KRAB expression resulted after induction with pristinamycin. Importantly, once the network balance had been tipped toward one state, the change became self-perpetuating and, following removal of the initial inducer, was not lost. This was in contrast to isogenic control experiments using separate Pip-KRAB and E-KRAB systems where expression levels markedly decreased following inducer removal. In addition to self-sustainability, it was also demonstrated that the system was reversible, and that the expression profile could be repeatedly switched between expression states over a two-week period.

These two characteristics, sustained expression stability and reversible switching, are also key requirements for epigenetic imprinting or memory that occurs when differential expression levels are imprinted and passed to subsequent cell generations well after the original signal generating that expression level has been removed. Many natural epigenetic switches have been characterized where their role has been implicated in coordinating diverse processes such as cell fate and memory [27], plant development [28], and lysogeny [29,30]. In this case, the synthetic mammalian switch provides one possible model for how epigenetic imprinting may occur at the transcriptional level in multicellular organisms. Beyond this, however, the toggle switch may also have important therapeutic applications. Classical transcription control systems operate in a dose-dependent manner and therefore require the on-going presence of regulating molecules to sustain transgene expression levels. Prolonged exposure to regulating molecules (e.g., antibiotics) can be associated with clinical ramifications such as the selection of pathogen resistance [31] and the accumulation of antibiotics in bone and teeth [32]. A self-sustaining, yet reversible, genetic network that requires only a transient stimulus to establish a steady state may provide an attractive means of overcoming such considerations.

15.3.4 Hysteresis

In a typical bistable switch movement between expression levels occurs in a quasi-discontinuous manner once a controlling stimulus crosses a specific threshold. This threshold is the same regardless of the direction in which the switch is being moved. A refinement on this switch is where the threshold required to move the switch in one direction is different to the threshold required to move it in the other direction. Thus, the threshold required to flip the switch depends on the starting state of the switch. This phenomenon, which can occur at molecular or macroscopic levels, is known as hysteresis [33]. To use a nonbiological example, traffic jams often exhibit hysteresis because the car density required to alleviate the traffic jam is less than the density that initially caused the jam. In a genetic network, a switch exhibits hysteresis when a different concentration of inducer is required to shift a system from one state to another than is required for the reverse shift [18,22,24,29,34,35]. Hysteretic behavior has been observed in several natural examples including the control of lactose utilization in *E. coli* [33], and ensuring unidirectional cell-cycle progression in eukaryotes [36]. A significant benefit of a hysteretic system is its inherent ability to buffer against modest changes in the inducing molecule. Thus, to switch a system from one state to another and then to back again requires a far greater change in inducer levels than in an equivalent typical bistable switch. Such devices could have broad potential for applications in which the input signal is prone to minor fluctuations but for which a constant all or nothing expression status is required.

Using a positive autofeedback mechanism and competitive transcriptional mechanism, a synthetic hysteretic switch has been constructed in mammalian cells (Fig. 15-7) [37]. The system used a tetracycline-dependent transactivator (TetR-VP16), which induces its own transcription via positive feedback together with a reporter gene (SEAP), as well as a competing erythromycin-dependent

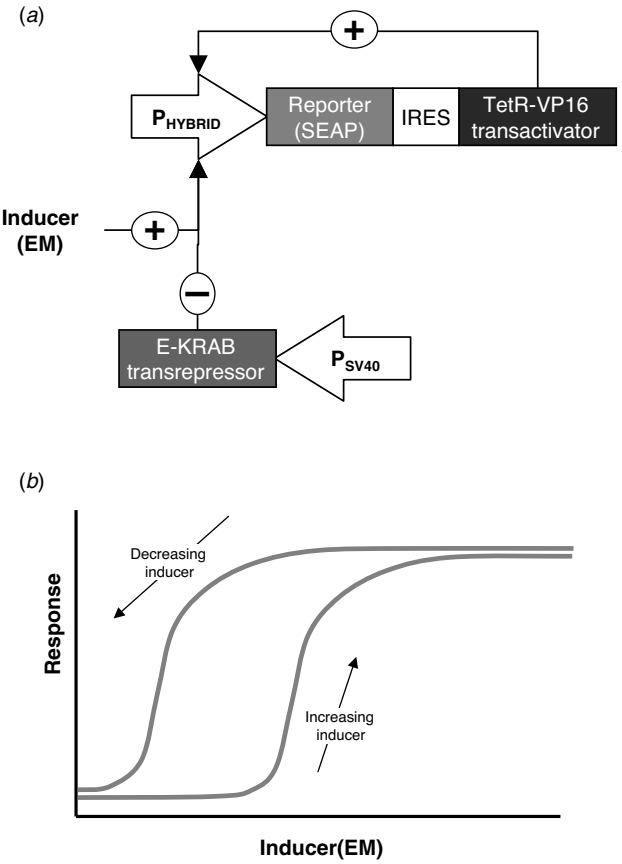

Figure 15-7 (a) Genetic design and (b) response profile of an engineered mammalian hysteretic switch.The hysteretic eukaryotic switch is based upon a chimeric promoter (P_{hybrid}) that drives expression of a SEAP (human placental secreted alkaline phosphatase) reporter gene and, via an IRES, the tetracycline-dependent transactivator (TetR-VP16). P_{hybrid} is responsive to both TetR-VP16, which establishes a positive autofeedback loop, as well as the erythromycin-responsive transrepressor (E-KRAB), which is independently expressed from a separate constitutive promoter (P_{SV40}). E-KRAB inhibits P_{hybrid} in an EM dose-dependent manner whereby a higher concentration of EM is required to switch the system from OFF to ON than is required to return the system from an ON to OFF state. The switching behavior of the network is therefore dependent upon the network's EM cultivation history [37].

transrepressor (E-KRAB), which was capable of inhibiting the TetR-VP16 mediated positive feedback. The hysteretic behavior of the synthetic network results from the competitive interaction of TetR-VP16 and E-KRAB for an engineered hybrid promoter (P_{hybrid}) that was responsive to both TetR-VP16 and E-KRAB. At low-EM concentrations E-KRAB binds P_{hybrid} and inhibits both TetR-VP16 positive feedback and SEAP expression (i.e., an OFF configuration). At high-EM concentrations, disassociation of E-KRAB from P_{hybrid} enables TetR-VP16 mediated transactivation resulting in positive autofeedback and high SEAP expression (i.e., an ON

configuration). The observed hysteretic behavior occurs due to the interaction at intermediate EM concentrations where the prevalence of E-KRAB-mediated inhibition versus TetR-VP16-mediated positive feedback depends upon historical EM concentration. A high historical EM concentration means a high level of TetR-VP16 is already present, which therefore requires greater E-KRAB activity, and correspondingly lower EM concentration, to drive the expression state from ON to OFF. The converse applies for low historical EM concentrations where minimal to no TetR-VP16 is present. In this case a significantly higher EM concentration is required before TetR-VP16 autoexpression becomes self-sustaining. For TetR-VP16 to outcompete E-KRAB full derepression of all E-KRAB activity is required which is achieved through a relatively much higher EM concentration. In this process the level of active TetR-VP16, and therefore the extent of positive feedback, acts as a molecular ''memory'' of the historical EM concentration of the system. If the extent of positive feedback is reduced, for example through tetracycline addition, which reduces the level of active TetR-VP16 in the system, then the EM concentration required to switch the system between ON and OFF configurations begins to resemble a classical graded profile thereby removing the hysteretic effect. While it was not possible to test using the constructed system, it is plausible that if the positive feedback within the system could be rendered sufficiently strong, then even the complete removal of EM would not be sufficient to enable E-KRAB to outcompete TetR-VP16. In such an event the system would exhibit irreversibility.

The importance positive feedback mechanisms has long been recognized as essential for many cellular processes and is increasingly being identified in natural biological systems, including signaling pathways [29]. For example, the maturation of the *Xenopus* oocytes involves the p42 mitogen-activated protein kinase (MAPK) and the cell-division cycle protein kinase Cdc2, which form positive autofeedback loops. Both mediators generate an irreversible switch-like response following transient stimulation with the steroid hormone progesterone. If the feedback loops are selectively disrupted using specific inhibitors, progesterone-induced maturation can still occur, however, the presence of progesterone must be actively maintained. Thus, following disruption of positive feedback the ability of the system to ''remember'' a transient signal is compromised [35,38]. Using synthetic genetic networks it has now also been possible to empirically demonstrate the role of feedback mechanisms in ensuring expression stability.

The synthetic networks described above show that either a single positive feedback loop or a double negative feedback loop can result in bistability. Future work in designing synthetic systems, as well as the study of naturally occurring networks, may yet identify other mechanisms for switching and the generation of sustainable responses to transient stimuli.

15.3.5 Oscillator

Expression stability is a common element of all the aforementioned networks. Dynamic instability, in which transcriptional components are in a constant state of flux, can result in an equally exciting behavior characterized by periodic, as opposed to

stable, expression. Where such periods are of a consistent period and amplitude, and require minimal to no external stimuli, the resulting behavior is oscillatory in character. Such behavior is found in a wide range of natural systems from archaebacteria to eukaryotes with the most well-known example being the circadian rhythm [39]. In humans, processes such as body temperature modulation, endocrine production and release, and immune responses exhibit circadian oscillations [40]. Circadian clocks have been proposed to consist of autoregulatory loops that use transcriptional feedback and high protein decay rates to maintain 24 h periodicity [41–43]. Similarly to the creation of expression stability, several synthetic approaches utilizing transcriptional feedback have successfully resulted in the creation of oscillatory behavior.

15.3.5.1 Bacterial Oscillator (“Repressilator”) Elowitz et al. constructed a plasmid-based synthetic oscillator in *E. coli* (termed the “repressilator”) from three common bacterial transcriptional repressor systems that are not part of any natural biological clock mechanism [44]. The three repressor systems were interconnected such that they formed a cyclic negative feedback loop or “daisychain” (Fig. 15-8). This configuration produced oscillating levels of each repressor protein. A GFP reporter gene, carried not only on a separate plasmid but also under the control of a promoter induced by one of the repressors, provided a readout of oscillations for that repressor. A mathematical model was again used to predict the parameters required for steady and repeated oscillations. Key requirements included strong promoters with tight induction characteristics and minimal leakiness, cooperative repression, and comparable protein and mRNA decay rates. The first requirement was achieved using engineered *E. coli* promoters that exhibited similar strong induction profiles. To reduce repressor protein half-lives a bacterial destruction tag was fused to the 3’-end of each repressor. The reduction in repressor half-lives approximately from 60 to 4 min ensured that protein decay rates were similar to mRNA decay rates of a 2 min. Finally, to ensure a cyclical readout was technically observable, the GFP reporter gene was also engineered to reduce its effective half-life.

Initial attempts focused on determining whether oscillations could be observed across a population of cells. Using a transient dose of IPTG, an inhibitor of one of the repressors (LacI), an attempt was made to synchronize the population at a common point. While a single damped oscillation was subsequently observed, the lack of any mechanism to ensure the cells remained synchronized, meant that no further oscillations could be discerned at a population level. Although not performed in this case, cell synchronization could potentially be achieved by coupling the oscillating network to a periodic process that is intrinsic to the cell [45], or by using a quorum-sensing mechanism or other intercell signaling to ensure that cells remain synchronized [46–48]. Nonetheless, by following individual cells it was possible to observe repeated oscillations (Fig. 15-8). Despite high variability between cells, which were attributed to random stochastic influences, oscillatory periods of approximately 160 min were observed. Given *E. coli* cell division times of 50–70 min, the almost threefold longer oscillatory periods indicated that the state of the network could be successfully passed to progeny cells.

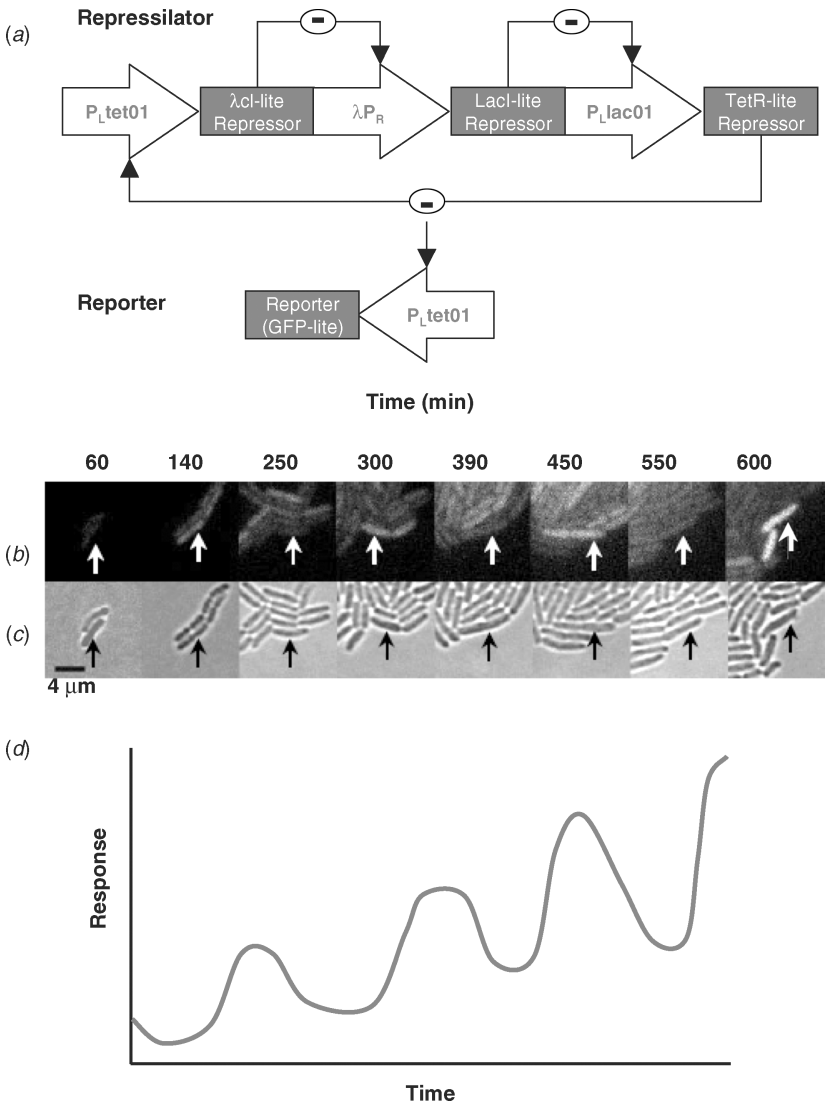

Figure 15-8 (a) Repressilation in bacteria: Genetic architecture of oscillatory network, (b) fluorescent and (c) bright-field snapshots of individual cells, and (d) GFP expression profile. The bacterial oscillatory network was constructed from three bacterial repressor systems arranged in a cyclical negative feedback loop. The first repressor protein, λcI, inhibits transcription of the second repressor protein LacI (from λP_R), which inhibits transcription of the third repressor protein TetR (from $P_L$lac01), which in turn inhibits expression of the first repressor (from $P_L$tet01) thereby completing the feedback loop. A reporter gene (GFP) under the control of a separate Tet-responsive promoter ($P_L$tet01) was used to assess oscillating TetR levels. By engineering short repressor and reporter half-lives (designated as lite) a dynamic unstable state was achieved in which TetR repressor levels cyclically rose and fell as evidenced through direct observation of individual cells and by GFP timecourse [44].

Shortly thereafter, the construction of another bacterial oscillator made from noncircadian components was reported [34]. In this case, it was constructed using a combination of positive (an ''activator'' module) and negative (a ''repressor'' module) feedback mechanisms (Fig. 15-9). Critical to producing a dynamic unstable outcome was the use of a hybrid promoter, capable of responding to both an activator and repressor, which effectively integrated the positive and negative feedback modules [49]. The resulting competitive interaction resulted in the ''burst'' like generation of activator and repressor proteins, which progressively smoothed over time. When coupled to a reporter system capable of measuring repressor levels, the result was a series of oscillations that progressively damped over time. However, in contrast to the bacterial repressilator developed by Elowitz et al., it was possible to synchronize a population of cells, via transient inhibition of the repressor, and observe up to four damped oscillations across the entire population. Oscillatory behavior, exhibiting periods close to 10 h, could be observed in continuous culture for up to 70 h again indicating that the network could be passed to subsequent progeny and that it was much more resistant to intrinsic noise than the ''repressilator.'' Through mathematical modeling it was predicted that the key parameter causing damped, as opposed to sustained, oscillations in the system was the respective differences in half-lives between the activator and repressor proteins. Although not experimentally tested it was predicted that sustained oscillations could be achieved by increasing the half-life of the repressor whilst decreasing that of the activator.

15.3.5.2 A Mammalian Oscillator? Unlike other expression functions, the development of a synthetic eukaryotic oscillator has not yet mirrored the creation of the bacterial equivalent, although given the pattern for these developments, it will not be surprising to see the emergence of a synthetic eukaryotic network in the near future. However, given the intense interest in understanding the mechanisms responsible for the natural circadian clock, it is also not surprising that attempts have been made to create a synthetic clock from actual clock components.

Using the core set of positive and negative regulatory elements common to all known circadian mechanisms, including the *cryptochrome* genes CRY1 and CRY2, the *period* genes PER1, PER2, and PER3, and the positive transcription factors BMAL1 and CLOCK [50–52], an attempt has been made to artificially engineer an oscillatory clock [53]. Among these components, BMAL1/CLOCK are positive regulators of CRY and PER proteins that, upon accumulation over a specific threshold, translocate to the nucleus where they negatively inhibit not only their own expression but also BMAL1/CLOCK. In this model, BMAL1/CLOCK mediated transcriptional inhibition is eventually relieved by PER and CRY degradation [54]. In the synthetic approach, BMAL1 and CLOCK expressions were placed on a tetracycline inducible ''positive'' regulation construct while PER, CRY, and a destabilized reporter gene were placed on a ''negative'' regulation construct in which their expression was under the control of a BMAL1/CLOCK/PER/CRY responsive promoter. Theoretically, turning the system ON by withdrawing tetracycline leads to BMAL1/CLOCK expression that subsequently drives expression from the negative regulation construct. Accumulation of PER/CRY eventually leads to autofeedback inhibition of the negative regulation

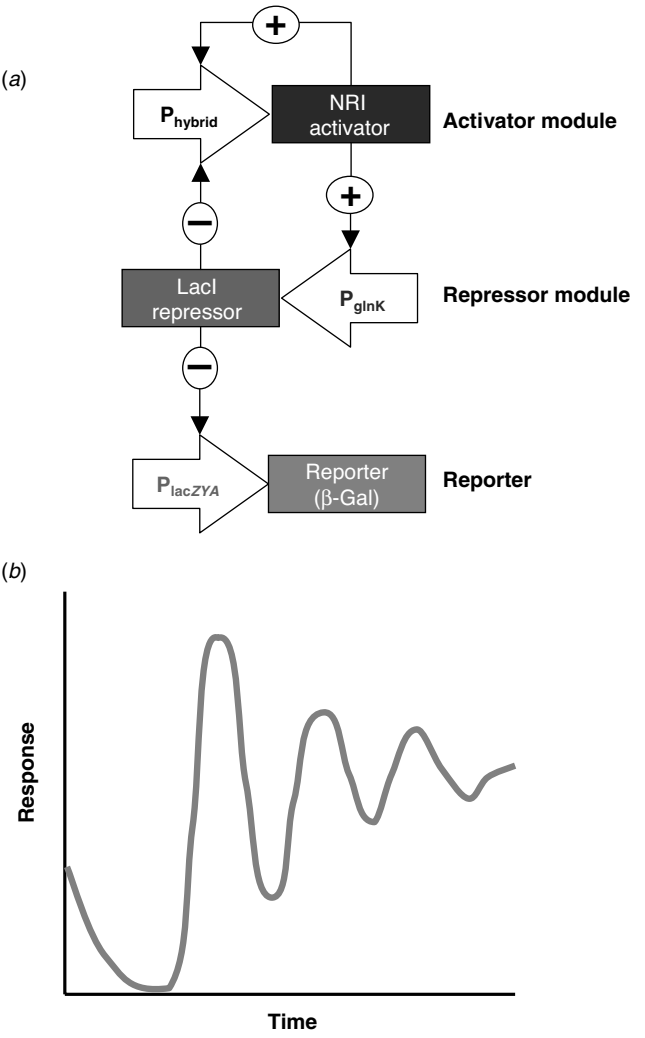

Figure 15-9 (a) Genetic design and (b) response profile of bacterial oscillatory network using positive and negative feedback mechanisms.A bacterial oscillatory network constructed from an activator and repressor module. The activator module forms a positive autofeedback loop in which the NRI transactivator activates its own expression from P_{hybrid}—a modified glnALB promoter (P_{glnk}) engineered to include LacI operator sites in addition to normal NRI operator sites. NRI also activates expression, via $P_{gln}k$, of the LacI repressor module which in turn repressors expression of NRI via P_{hybrid}. A reporter construct consisting of β-galactosidase and employing a LacI repressible promoter was used to assess oscillating levels of LacI repressor. Following synchronization with a transient pulse of IPTG (an inhibitor of the LacI repressor), up to four damped oscillations were observed across a cell population [34].

construct that, to complete the cycle, is relieved by eventual degradation of PER/CRY. In practice,this system was not capable of producing sustained oscillations, yet it did nevertheless exhibit a single cycle of a clock-like oscillation, which at a minimum establishes the possibility that homologous regulatory components can be used for synthetic constructions and that the design and creation of a successful mammalian clock will necessitate the incorporation of some kind of feedback mechanism. This latter conclusion is supported by recent experimental analysis of the mammalian clock system where directed disruption of CRY-mediated transcriptional autorepression resulted in arrhythmic phenotypes in both single- and multicell populations [51].

The aforementioned networks indicate that transcriptional feedback and feedforward processes are ubiquitous mechanisms for ensuring controlled expression whether that output is stable, binary, toggle, hysteretic, or even for periodic oscillating behavior. The further creation and characterization of synthetic networks will hopefully determine whether feedback is a minimal requirement for all networks or whether any other mechanisms could produce novel functional expression forms.

Alongside developments into unique expression states have been the concomitant development of novel means of integrating signals—so called ''input'' functionality. This has included the serial linking of transcriptional control systems to form transcriptional cascades, the creation of electronic circuit emulating logic gates, and the development of sophisticated sensors enabling cell-to-cell communication.

15.3.6 Transcriptional Cascades

Initial attempts in constructing regulatory cascades involved the construction of a two-level cascade using the $TetR_{OFF}$ and Lac_{ON} systems in mammalian cells [55]. In this simple system, the TetR-VP16 transactivator was constitutively expressed and, via a TetR-VP16 responsive promoter, drove the expression of a LacI repressor. LacI in turn inhibited expression of a reporter gene, via a LacI-inducible promoter. In this case, reporter gene expression could occur either in the presence of tetracycline, which prevents LacI expression, and/or in the presence of IPTG, which inhibits LacI repression of the reporter gene. This pioneering system established the basis for interconnecting gene control systems and successfully enabled the tight induction characteristics of the Tet_{OFF} system to be used to for an ON-type system (i.e., addition of tetracycline results in reporter gene expression), which in their native form (i.e., TetR-KRAB) do not typically exhibit such tight regulation. However, the high cytotoxicity of IPTG in mammalian cells is likely to prevent any clinical application of this technology.

In a very similar approach, Imhof et al. constructed a regulator network consisting of an engineered tetracycline-dependent transrepressor (TetR-KRAB) that controlled the expression of a Gal4-VP16 transactivator, which in turn controlled its own expression as well as a highly cytotoxic reporter gene (diptheria toxin A) [56]. Gal4-VP16 is an OFF-type system but exhibits typical residual leaky transcriptional control. Tight repression of Gal4-VP16 by TetR-KRAB ensured no reporter gene expression under noninduced conditions, whereas addition of tetracycline resulted in derepression of Gal4-VP16, subsequent autoexpression of further Gal4-VP16, and subsequent strong reporter gene expression. In this manner, a cascade was

used together with a regulatory feedback loop to amplify the window of transgene regulation resulting ultimately in extremely tight transcriptional control.

15.3.6.1 Multilevel Gene Control Most classical transcriptional control systems exhibit sigmoid-shaped dose–response characteristics where the range within the system flips between ON and OFF states is relatively narrow. As previously which mentioned, this is predominantly due to the transcriptional cooperativity inherent to most gene control systems. One consequence is that most current transcription control systems operate in an all or nothing manner (i.e., ON or OFF) and are not reproducibly capable of intermediate levels of adjustment. It is conceivable that future gene therapy applications will require precise dosing of therapeutic genes in much the same way that dosing of pharmaceuticals is critical to their successful application. All or nothing control mechanisms may therefore be of limited use. By combining several typical ON/OFF mechanisms in a network configuration, it has been possible to construct a gene control system where a target gene can be accurately and repeatedly titrated to intermediate levels [57].

Multilevel transgene control was achieved through the cascade arrangement of three heterologous control systems: the tetracycline (Tet_{OFF}), macrolide (E_{OFF}), and streptogramin (PIP_{OFF}) systems (Fig. 15-10). As these systems and their inducers (tetracycline, erythromycin, and pristinamycin, respectively) exhibit minimal to no cross-interference, it was possible to connect them in a linear type fashion whereby each system acts as the activator of the next system. All of the selected systems were OFF-type systems in which transcription is active in the absence of inducer and repressed following addition of inducer. Here, addition of each respective inducer prevents transcription of the next component in the cascade. However, as all of these systems exhibit minimal residual expression following addition of inducer (referred to as ''leakiness''), there is nonetheless some activation of lower levels in the cascade. The impact of this leakiness on total expression levels depends upon the point in the cascade at which it occurs. Thus, at ''upstream'' points within the cascade, transcriptional leakiness is amplified by latter stages thereby limiting the extent of overall OFF switching. For ''downstream'' interventions within the cascade there is minimal opportunities for transcriptional leakiness to be amplified. The result is that upstream interventions have less impact on overall expression than downstream interventions. Using different inducers it is possible to select the desired intervention point as each inducer affects a different point in the cascade. Thus, expression levels of 100 percent (no cascade intervention), 70 percent (intervention at first level of cascade), 40 percent (intervention at second level), and close to 0 percent (intervention at third and final level) of a target reporter gene were possible. This genetic network demonstrated that the typical ON/OFF switching characteristics of current control systems, together with residual inherent leakiness, could be exploited to produce a system capable of intermediate expression levels in response to up to three different inputs [57].

15.3.6.2 Regulation Sensitivity In a similar experimental approach, but with a different outcome, up to three bacterial transcriptional repressors were linked in a linear cascade [58]. Unlike the heterologous systems employed above, homologous bacterial

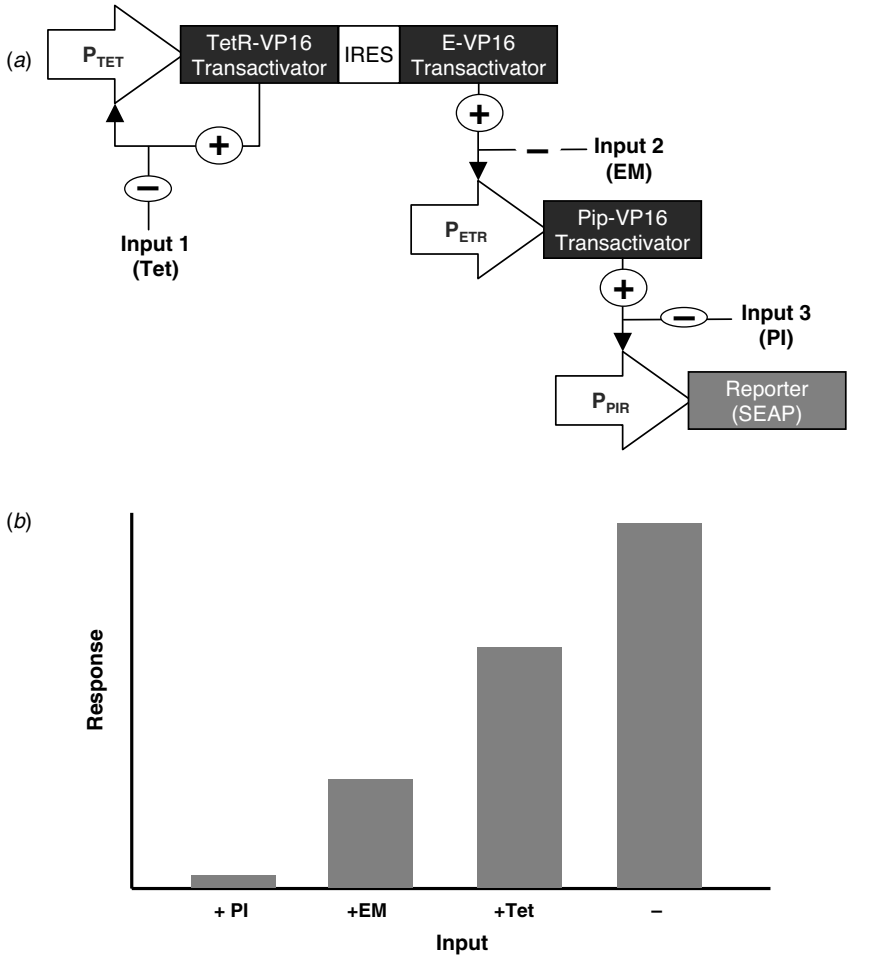

Figure 15-10 Network design (a) and regulation performance of a synthetic mammalian three-level regulatory cascade.The three-level regulatory cascade consists of three heterologous interconnected gene transcription systems. The tetracycline responsive promoter (P_{hCMV^*-1}) drives a dicistronic expression unit encoding the tetracycline-dependent transactivator (TetR-VP16) and, via an IRES, the macrolide-dependent transactivator (E-VP16). E-VP16 subsequently drives expression, via a macrolide-responsive promoter (P_{ETR}), of a streptogramin-responsive transactivator (Pip-VP16). Finally, Pip-VP16 drives expression of the reporter gene human placental secreted alkaline phosphatase (SEAP) from a streptogramin-responsive promoter (P_{PIR}). The linear arrangement ensures that SEAP expression can be controlled from a number of levels. Shutting off expression at the top of the cascade, by inhibiting the autofeedback loop controlling TetR-VP16 expression with tetracycline (Tet), reduces overall expression to approximately 70 percent of maximum noninduced expression. Closing the cascade further downstream, by inhibiting E-VP16 with erythromycin, has a greater impact reducing total expression to approximately 30 percent. Finally, interventions at the bottom level of the cascade, through inhibition of Pip-VP16 with PI, reduces expression within the system to almost baseline levels [57].

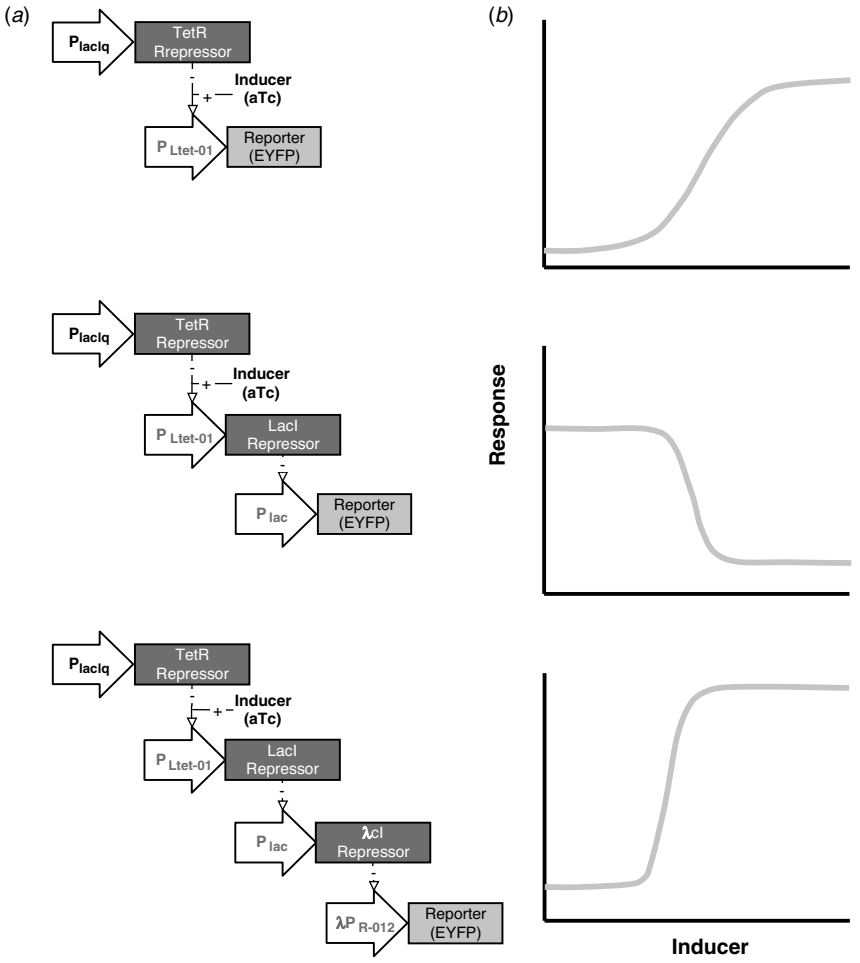

Figure 15-11 (a) Genetic architecture and (b) dose–response curves of single-, two-, and three-level transcriptional cascades. Transcriptional cascades were assembled using up to three bacterial repressors linked to each other in a linear fashion. In each case the tetracyline repressor (TetR) was constitutively expressed, induced with aTc, and system output measured by enhanced yellow fluorescence protein (EYFP) production. In the single-level cascade, TetR bound the $P_{Ltet\text{-}01}$ promoter where it directly repressed EYFP production. In the two- and three-level cascades, TetR repressed production of a second repressor (LacI), also from $P_{Ltet\text{-}01}$. In the two-level cascade, LacI repressed production of EYFP from P_{lac}. In the three-level cascade, LacI repressed production of yet a third repressor, (λCI) from P_{lac} which in turn repressed production of EYFP from $\lambda P_{R\text{-}O12}$. Dose–response curves for the three types of cascades reveal that the inducer range needed to effect a change between ON and OFF states narrows with the length of the cascade thereby increasing sensitivity to the inducer [58].

repressors exhibit much tighter regulation performance with virtually no leakiness. Hence, rather than creating multilevel gene control, the aim was to investigate the impact of multilevel cascades on the regulation performance of a typical bacterial repression system, the TetR system (Fig. 15-11). Three versions were compared; a single-level cascade—where TetR directly represses expression of a reporter gene, a two-level cascade—where TetR represses transcription of a second repressor which in turn controls the reporter gene, and a three-level cascade—where yet another repressor system was introduced between the second repressor and reporter gene.

Dose–response experiments indicated that the number of levels, or depth, of a cascade has a significant impact upon a number of regulation characteristics. First, the sensitivity of the cascade increases with the depth of the cascade. Thus, the system switches between low and high from a smaller range of input values. Second, the extent of noise within the system, as seen by variation in fluorescence across a population, while minimal at input ranges far from the transition region, increases with the length of the cascade. Deeper cascades serve to amplify the noise around the transition point presumably due to the extra number of transition points involved. This may limit the utility of adding even further cascade levels as additional increases in noise amplification around transition points may ultimately offset any further sensitivity gains. Third, the delay in the output response of the system increases commensurately with the depth of the cascade. This is to be expected and is largely the result of protein production and decay rates, and repression thresholds. Interestingly, there is evidence that time delays caused by regulatory cascades may actually be a design parameter required for many natural gene networks [59]. Database analyses of natural networks, which are involved in rapid and reversible gene expression in response to external stimuli (so called ‘‘sensory’’ transcriptional networks), reveal that such networks generally contain short regulatory cascades. Networks involved in slow and irreversible gene expression during development (so called ‘‘developmental’’ transcriptional networks) typically contain longer cascades.

15.3.7 Logic Gates

The expression output of many cell-based regulatory networks is often a logic response generated by one or more input signals. Due to their sigmoid-shaped dose–response curves, most gene control systems can be regarded as the genetic equivalent of an analog-to-digital converter. Their output is either ON or OFF across a wide range of inducer concentrations, except for a small concentration window where transitions between the two states occur. In this regard, the analogy between genetic networks and electronic circuitry is very compelling. This has led to the conceptualization of genetic networks as logic gates with switchboard-type truth-tables and schematic representations that directly mirror electronic circuit diagrams [60–62]. Adapting gene control systems to Boolean language, ON-type gene control systems represent IF type gates in the sense that expression results IF an input is present. Conversely, OFF-type gene control systems represent NOT type gates whereby expression results when an input is NOT present.

By utilizing several compatible heterologous gene control systems responsive to tetracycline, macrolide, streptogramin, and butyrolactone input signals, it has been

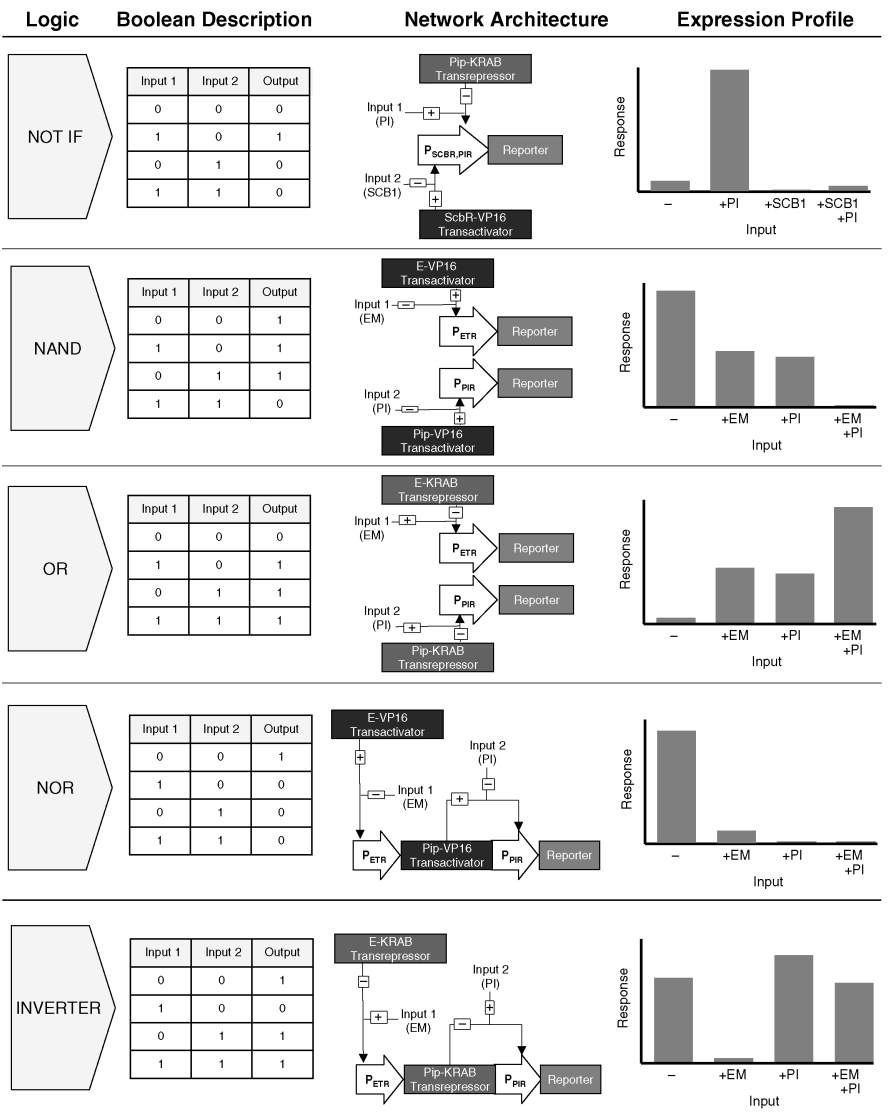

Figure 15-12 Boolean description, network architecture, and expression profile of five mammalian BioLogic Gates. All five mammalian logic gates were constructed from heterologous mammalian transcription systems. In the NOT IF gate, the butyrolactone-responsive transactivator (ScbR-VP16) and the streptogramin-responsive transrepressor (Pip-KRAB) are constitutively expressed and modulate expression of a reporter gene from a chimeric promoter ($P_{SCBR,PIR}$) containing operator sites for both ScbR-VP16 and Pip-KRAB. Input signals, 2-(1'-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide (SCB1) and/or PI result in disassociation of ScbR-VP16 and Pip-KRAB respectively. Expression only occurs when ScbR-VP16 is bound to the chimeric promoter and Pip-KRAB is disassociated therefore requiring the absence of SCB1 and presence of PI. For the NAND gate, both the macrolide-responsive transactivator (E-VP16) and the streptogramin-responsive transactivator (Pip-VP16) are constitutively expressed. Each transactivator binds its cognate promoter (P_{ETR} and P_{PIR}, respectively) which drive separate expression of two copies of

possible to design a range of eukaryotic logic circuits that follow strict Boolean logic in their integration of two input signals (Fig. 15-12) [63]. Hence, in the NOT IF gate, expression of a reporter gene occurs if and only if one specific input is present and the other input is absent. In the NAND gate, expression always occurs unless both inputs are present. The converse, where expression always occurs unless both inputs are absent, is reflected in the OR gate. The inverse, where expression occurs only when both inputs are absent is reflected in the NOR gate. Finally, the INVERTER gate represents the opposite of the NOT IF gate whereby expression always occurs unless one specific input is present and the other input is absent. Analogously to electronic circuit design some of these networks were constructed by linking elements in parallel while others were constructed by combining elements in series through the use of simple transcriptional cascades. These examples demonstrate that a considerable range of logical switches responding in unique ways to the same two input signals can be constructed from modular transcriptional control components. It is imaginable that such networks could be highly useful for gene therapy applications that require a particular response to highly specific inputs, which could vary depending upon the application.

Similar to electronic circuit design, the above switches were based on rational design principles. However, a number of other approaches have also been used to produce electronic-type circuit behavior, which produce a defined output in response to two inputs. Guet et al. used a combinatorial method involving prokaryotic transcriptional control systems that were randomly combined to generate a library of networks with varying connectivity [64]. From this library it was possible to isolate and characterize a range of diverse computational functions that produced unique phenotypes. While such an approach may yield unexpected network architectures for

the same reporter gene. Input signals, EM and/or PI, modulate transactivator activity respectively. Expression occurs when either or both transactivators are bound to their cognate reporter. The presence of both EM and PI are required to disassociate both transactivators to prevent expression. The OR gate is identical in design to the NAND gate but uses the transrepressor versions (i.e., E-KRAB and Pip-KRAB) of the macrolide- and streptogramin-responsive transcription control systems. Again, EM and/or PI modulate transrepressor activity respectively. In this case, expression is blocked only when both transrepressors are operator bound which only occurs when both EM and PI are absent. The NOR gate involves a short linear cascade between a constitutively produced macrolide-responsive transactivator (E-VP16) which drives the expression, via its cognate promoter (P_{ETR}), of the streptogramin-responsive transactivator (Pip-VP16) which in turn drives expression, via its cognate promoter (P_{PIR}), of a reporter gene. Modulation of transactivator activity is achieved through EM and PI, respectively. In this configuration, expression only occurs when E-VP16 is bound to its cognate operator and Pip-VP16 is disassociated from its cognate promoter therefore requiring the absence of both EM and PI. The final gate, the INVERTER, is identical in design to the NOR gate but uses the transrepressor versions (i.e., E-KRAB and Pip-KRAB) of the macrolide and streptogramin responsive transcription control systems. Again, EM and/or PI modulate transrepressor activity respectively. The only conditions under which expression will not occur are when E-KRAB is promoter disassociated and Pip-KRAB is promoter associated which occurs in the presence of EM and absence of PI. For each gate, the input and output characteristics of the Boolean description are reflected in the expression profile of the synthetic system [63].

a given function, the approach is not particularly amenable to forward engineering approaches that seek to design circuits that exhibit specifically required functions. In a related approach, Yokobayashi et al. combined rational design with an evolutionary approach to design specific circuits in *E. coli* [65]. Rational design based upon existing knowledge of well-characterized components was initially used to design a network with a specific function. Given that the synthesized network exhibited sub-optimal behavior, due to unexpected interactions and poor matching of network components, a directed-evolutionary approach was then used to fine-tune (or ''debug'') the system to obtain the required function. This was achieved through sequential rounds of localized random mutagenesis and recombination followed by phenotype screening. Subsequent sequence analysis of successful networks revealed that many changes, or ''solutions'', were capable of producing the desired phenotype. This could be manifested in changes which altered either protein-DNA or protein–protein interactions, but which nonetheless enabled superior biochemical matching of genetic components.

15.3.8 Sensory Networks

15.3.8.1 Signal Amplification To extend the electrical circuit analogy further, Karig and Weiss recently developed a highly effective signal-amplifier from prokaryotic bacterial control systems [66]. Their aim was to try and develop a means for detecting weak transcriptional responses that, despite being difficult to detect *in vivo*, are often involved in regulatory functions where only trace amounts of a gene product are required. In typical transcriptional studies aimed at determining the conditions under which a promoter is activated, a reporter gene is placed downstream of the promoter and assayed under varying conditions. However, where the promoter response is weak it is often not possible to discern any kind of activity. By placing a repressor cascade downstream of the promoter it was possible to amplify an otherwise undetectable promoter response. In their system, Karig and Weiss placed the λcI repressor downstream of several Rhl quorum sensing (qsc) promoters from *Pseudomonas aeruginosa*. By coupling the repressor to a fluorescent reporter, under the control of a $\lambda P_{(R\text{-}O12)}$ promoter, they were able to monitor the response of selected promoters to acyl-homoserine lactones (AHL). As λcI is a highly efficient repressor, even very low concentrations of λcI can completely repress $\lambda P_{(R\text{-}O12)}$ thereby altering the fluorescent reporter readout. The amplifying cascade allowed up to 100-fold differences in fluorescence to be observed, between AHL-induced and -noninduced conditions, for promoters whose responses were otherwise not detectable. Apart from illustrating a biological means by which weak transcriptional responses can be amplified, the amplifying circuit could potentially be useful for a number of applications including the detection of trace toxins or molecules.

15.3.8.2 A Band-Detection Network One can imagine it would be useful for a range of applications to design an input mechanism that can respond to an inducer within a given concentration range, or perhaps one which is capable of a transient response when a progressively increasing inducer reaches a threshold concentration.

In a series of innovative synthetic constructions Basu and colleagues recently created synthetic networks capable of such behavior in *E. coli* [67–69].

The key requirements for band-detection network are the design of modular components that enable the detection of a low-threshold, a high threshold, and a means of integrating the two thresholds. In this case, this was achieved by exploiting differences in repressor activities, and by linking several bacterial repressor systems (Fig. 15-13) [67]. Guided by mathematical analysis the band-detection thresholds were engineered by combining high-detection and low-detection componentry.

For both components the initial input was the same and was represented by the extent of LuxR activity—a bacterial activator that is activated by the inducer compound AHL. In the high detection componentry, the LuxR activator drives expression, via its cognate promoter, of a weakened secondary repressor, $LacI_{M1}$ which if present in sufficiently high quantities prevents expression of a reporter gene from the P_{lac} promoter. Thus, the boundary of the high threshold is determined by the amount of AHL required to produce enough $LacI_{M1}$ to repress the P_{lac} promoter that in turn depends upon the relative activity of the $LacI_{M1}$ repressor. The low detection componentry also relies on the LuxR activator, but to express the strong λcI repressor. This in turn is coupled via a transcriptional cascade to production of wild-type LacI, which, like the $LacI_{M1}$ repressor, also represses expression of the reporter gene. In this case, the boundary of the low threshold is the lowest amount of AHL required to prevent λcI expression thereby enabling the wild-type LacI repressor to be fully expressed resulting in reporter gene repression. It is only between the two thresholds that both the high and low detection componentry fail to repress the reporter gene. Hence, the relative activity of the $LacI_{M1}$ repressor and the AHL concentration that results in λcI expression are the two key components that determine the size and location of the band-detection characteristics. By altering the activity of the $LacI_{M1}$ repressor, Basu et al. were able to create three versions of the band-detection network each with differing upper detection limits.

15.3.8.3 A Pulse-Generating Network Basu et al. also utilized the above bacterial componentry to develop a network capable of producing a transient pulse when exposed to increasing concentrations of AHL [69]. The pulse-generating network produces output when a threshold concentration of increasing AHL is reached, and then through a feedforward mechanism shuts down reporter expression regardless of whether AHL concentration continues to rise or fall [70]. In this network AHL again activates LuxR, which in this case is constitutively present. Activated LuxR activates both a destabilized λcI repressor as well as directly activating reporter gene expression via a chimeric hybrid promoter responsive to both LuxR and λcI. Hence, increasing levels of AHL initially trigger both reporter and λcI expression. Following a delay, λcI accumulates to a sufficient extent where it eventually shuts down reporter expression. Like the band-detection network the pulse-generating network provides important insights into how pulse-generating behavior could occur in natural systems.

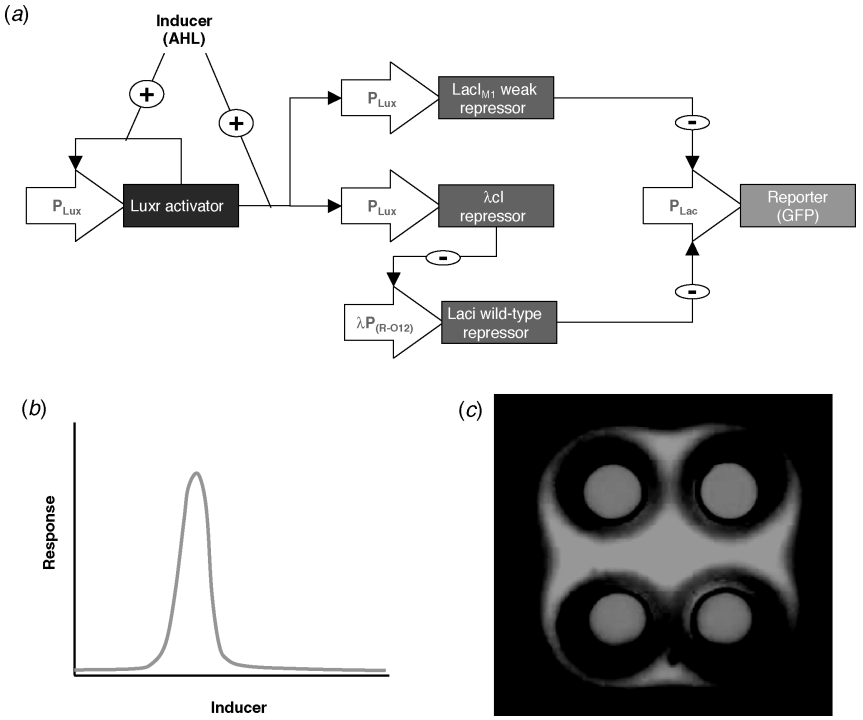

Figure 15-13 (a) Genetic architecture, (b) dose–response profile, and (c) pattern formation of a band-detection network.(a**)** In the band-detection network acyl-homoserine lactone (AHL) binds LuxR, an AHL-dependent transcriptional activator, which is produced in an autoregulatory manner from a P_{LuxR} promoter. LuxR also drives (from P_{LuxR} promoters) the expression of a λcI repressor and a weakened form of the LacI repressor ($LacI_{M1}$). The λcI repressor is coupled to a further regulatory cascade which represses the expression of wild-type LacI from a $\lambda P_{(R\text{-}O12)}$ promoter. Both the weakened and wild-type LacI repress expression, to a different extent, of a green fluroscence reporter gene (GFP) from a P_{Lac} promoter. At low AHL concentrations, LuxR is not active such that only basal levels of both $LacI_{M1}$ and λcI repressors are produced. The absence of λcI ensures that wild-type LacI is fully expressed which consequently represses GFP expression. At high AHL concentrations, the LuxR activator drives both high $LacI_{M1}$ and λcI expression. The presence of λcI ensures that wild-type LacI is completely repressed. However, as sufficiently high concentrations of $LacI_{M1}$ are expressed, GFP expression remains nonetheless repressed. It is only at intermediate concentrations of AHL that a balance is reached between sufficiently low expression of $LacI_{M1}$ to prevent $LacI_{M1}$-mediated repression of P_{Lac}, and sufficiently high expression of λcI to prevent LacI expression and consequent LacI-mediated repression of P_{Lac}. At this point insufficient repression from either LacI repressor results in GFP expression. **(**b**)** In an AHL dose–response curve, GFP expression is only observed within a band of AHL concentration. **(**c**)** If AHL is chemically produced and allowed to diffuse from a defined set of ''sender'' cells (exhibiting red fluorescence) placed within a lawn of ''receiver'' cells containing the band-detection network, the resulting AHL gradient produces a distinctive green fluorescence pattern based upon the spatiotemporal location of the receiver cells to the sender cells [67].

15.3.8.4 Cellular Cross Talk and Intercell Communication The band-detection and pulse-generation networks developed by Basu and colleagues have been successfully used to generate spatiotemporal differentiation patterns that, much like natural pattern formation, rely upon cell-to-cell communication and signal transduction networks [67,69]. In initial work, it was demonstrated that ''sender'' cells engineered to produce AHL could influence ''receiver'' cells endowed with synthetic networks capable of responding to AHL, which has diffused from the sender cells [71]. In pattern formation experiments cell-to-cell communication was commenced from ''sender'' cells, which produced an AHL concentration gradient. In one set of examples, receiver cells containing the band-detection network responded to the chemical gradient and at intermediate distances from the sender cells expressed their reporter gene in accordance with the AHL detection thresholds within their band-detection network (Fig. 15-13). Such cell-to-cell communication or cellular cross talk could be engineered to result in a range of patterns, and by altering the thresholds of the band-detection network and using different fluorescent reporter genes an impressive array of multicolored patterns and shapes could be produced. In addition to representing a sophisticated genetic network, the formation of patterns from a synthetic network together with cellular communication represents a significant step toward reproducing and understanding natural developmental processes. In addition to pattern formation, intercellular communication could also be used to ensure synchronization of cellular populations. Using *E. coli* as a model system it has been demonstrated that synthetic gene networks can be used to engineer an artificial quorum-sensing mechanism that utilizes a common cellular metabolite [72].

15.4 SEMISYNTHETIC NETWORKS

The majority of synthetic genetic networks built and characterized to date have utilized external signals to create a desired function. To reach their therapeutic potential, however, it will be necessary to design networks that are capable of responding not only to external signals but also to endogenous or physiological signals. Hence, one can imagine sophisticated networks that independently provide a therapeutic outcome in response to pathological signals, and can also be overridden or altered through external modulation should the need arise. While still in their infancy, several systems integrating physiological signals—so called ''semisynthetic'' systems—have already been developed.

In *E. coli*, semisynthetic systems have been designed, which interface various physiological inputs into a bacterial toggle network thereby producing a sustainable switch-like response to a transient physiological input [73]. A DNA damage sensing network was constructed by interfacing the SOS pathway to a bacterial toggle. The SOS pathway detects single-stranded DNA following DNA damage by activating RecA coprotease. Activated RecA subsequently cleaves the λcI repressor in the toggle circuit causing derepression of the $\lambda P_{R\text{-}O12}$ promoter, and a sustainable switch to high LacI production (the other repressor in the toggle circuit). If LacI production is linked to a fluorescent output, the system can detect and retain a memory of transient

DNA damage. In an alternate application, if the fluorescent reporter gene is substituted for a biofilm producing gene, transient DNA damage can induce the cells to commence biofilm production. In a separate example, the transgenic AHL quorum sensing pathway was interfaced to the toggle circuit. When AHL reached sufficiently high levels, (e.g., if cell density reaches a critical density), the AHL-dependent activator LuxR repressed the P_{Lac} promoter of the toggle thereby leading to high λcI expression. This semisynthetic system is capable of producing a sustainable output once cell density reaches a critical threshold.

In other prokaryotic systems, Farmer and Liao developed a feedback controller in *E. coli* in which the expressed genes are key enzymes in the lycophene biosynthesis pathway [74]. By engineering the genes to be under the control of a physiological metabolite that is present during periods of high glycolytic flux, it was possible to coordinate lycophene production with the energy status of the cell thereby preventing metabolic imbalance and suboptimal productivity. Using glycolytic flux as a physiological cue Liao and colleagues have also developed an oscillatory network that is coupled to *E. coli* host metabolism (termed the ''metabolator'') [75]. In this system, a steady state is dependent upon the relative state of two metabolic ''pools'' which under high glycolytic flux result in instability in the engineered network with consequent oscillations.

Progress has also been made in developing mammalian semisynthetic systems. The mammalian oxygen response system, in which a specific set of endogenous genes is induced in response to low oxygen levels (e.g., VEGF), relies upon the translocation of hypoxia-induced factor 1 alpha (HIF-1α) to the nucleus where through a series of interactions it activates expression from promoters containing hypoxia-response elements (HRE). Under normoxic conditions, HIF-1α is rapidly degraded thereby preventing the low-oxygen response [76,77]. A semisynthetic network has been created by coupling the HIF-1α response system to a mammalian heterologous regulatory cascade resulting in multilevel gene control that can be influenced by endogenous signals (i.e., oxygen levels) as well as external signals (Fig. 15-14) [78]. By combining three inputs, it has been possible to produce six distinct expression states depending upon the combination of signals used.

While representing the first steps toward the therapeutic application of synthetic networks, a major challenge remains to find and/or preferably design transcription control systems that not only detect changes to a specific endogenous inducer but also detect changes within a specified concentration range. The systems constructed to date have largely relied upon serendipity and have sufficed as a proof of concept. Yet to reach their true potential, one will need to find means of detecting and interfacing changes to pathologically relevant molecules.

15.5 THE INFLUENCE OF ''NOISE''

A major influence upon the fidelity and function of both synthetic and natural genetic networks is noise. Noise is evidenced by high fluctuation in expression levels, which if sufficiently high enough may produce very different network outcomes both within

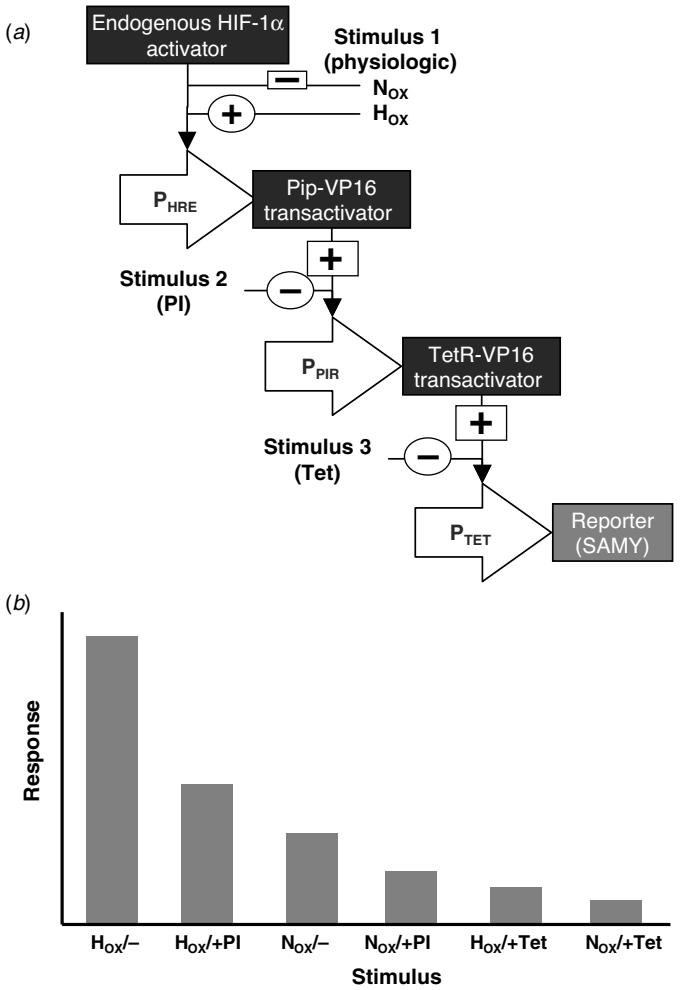

Figure 15-14 (a) Genetic layout and (b) response profile of a mammalian semisynthetic regulatory cascade.The semisynthetic cascade is triggered by endogenous HIF-1α that, under hypoxic conditions (H_{OX}), is mobilized to the nucleus where it binds and activates a synthetic promoter containing hypoxia-response elements (P_{HRE}). Under normoxic conditions (N_{OX}) HIF-1α is rapidly degraded to undetectable levels. Activation of P_{HRE} sets off a transcriptional cascade of two heterologous transcription systems; the streptogramin-responsive transactivator Pip-VP16 which upon expression binds its cognate promoter (P_{PIR}) leading to expression of the tetracycline-responsive transactivator TetR-VP16, which subsequently binds its cognate promoter (P_{TET}) leading to expression of a SAMY *(Bacillus stearothermophilus* derived secreted α-amylase) reporter gene. In addition to sensing physiologic oxygen levels via the HIF-1α activator, the system is also responsive to PI which interrupts the cascade at Pip-VP16, and tetracycline (Tet), which interrupts the cascade at TetR-VP16. Up to six expression levels can be produced by different combinations of the three inputs [78].

and across a cell population. Noise can be generated from a number of sources. In many circuits the genetic components required for gene expression, such as promoter sites, are typically present at very low copy levels. The result is that biochemical rates of transcription and translation are therefore correspondingly low and, compared to other cellular interactions (e.g., protein–protein interactions), occur relatively infrequently. Such infrequency can lead to large fluctuations, which due to their origination within the genetic circuit, are referred to as internal or intrinsic noise. Noise can also be generated externally or extrinsically of the circuit not only through stochastic variation in cellular components required for gene expression (e.g., polymerase, transcription, translation factors, and so on) but also through environmental or global changes that impact all gene activity (e.g., cell division). Given the modular nature of artificial gene networks, and the ability to rationally design them from well-understood components, it has been possible to gain insight into how noise is created, how it is propagated through a network, and finally instances where the existence of noise is in fact crucial to the output function of a network [79]. A significant body of work has focused upon designing synthetic networks to experimentally test predictions relating to noise phenomena. Such insights have also been crucial for the later design of noise-tolerant networks [80–82].

In early work on prokaryotic systems, it was shown that both intrinsic and extrinsic sources contribute significantly to the generation of noise that places certain inherent limits on the precision of gene expression [83,84]. It was also shown that the lower the effective strength of a promoter, whether through reduced gene copy number, repression, or a different cellular environment, the greater the extent of noise [83]. Other work aimed at determining whether transcription or translation is the major point of noise creation suggests that differences in translational efficiency have a greater impact than transcriptional differences [85] and that translational differences can result in variation which persists long after intrinsic noise from transcription has decayed [86].

The design and characterization of toggles, oscillators, and regulatory cascades has led to insights into how noise is propagated through a network [80,82]. As previously described, the presence of a simple autofeedback mechanism can reduce noise in a genetic network [18] and this has certainly been suggested as a major function of both positive and negative feedback mechanisms [70,87]. Failures in such mechanisms have been attributed to certain disease states. For example, the transformed phenotype in tumor formation has in some cases been attributed to instability of autocrine positive feedback loops [88]. Network connectivity can also be a major cause of noise. Using a bacterial regulatory cascade, Pedraza and van Oudenaarden demonstrated that the connectivity of sequential network components can result in an a total variation that is greater than the variation intrinsic to the expression of each component gene [84]. This implies that variation in a cascade can be cumulative. Hooshangi et al. in characterizing differences in their three-level bacterial regulatory cascade (described above) also witnessed greater variability around input transition points [58]. Indeed, the extent of variation can be so significant in a cascade that variations in an upper-level cascade within a cell population can cause the population to display bistable expression states [49,89–91]. That stochastic fluctuation can be crucial for generation of

cellular phenotypes is becoming more and more evident. Noise can establish an initial asymmetry that, once propagated and amplified through a network, may result in phenotypic consequences that impact processes such as differentiation and disease [90,92,93].

15.6 CONCLUSION

All of the engineered genetic networks thus described have utilized at least some aspects of rational design to produce a behavior that is based upon the modular interaction of DNA sequences and regulatory proteins. By assembling molecular parts not normally associated with each other into different configurations, it has been possible to produce an already impressive array of robust network behaviors. As the number of available modules increases, and their kinetic parameters become better characterized, and our ability to model and predict their interaction continues to improve, it is inevitable that further novel and increasingly more sophisticated synthetic networks will be created. Existing synthetic networks have already provided important insights and confirmation of hypothesis on a range of natural phenomena such as importance of feedback mechanisms, of balanced genetic componentry, of regulatory cascades, and of noise to name a few. It can also be expected that engineered gene networks will have many important biotechnological and therapeutic applications all of which aim to manipulate cellular processes at the genetic level.

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