Regulatory circuit design and evolution using phage λ

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Bistable gene regulatory circuits can adopt more than one stable epigenetic state. To understand how natural circuits have this and other systems properties, several groups have designed regulatory circuits de novo. Here we describe an alternative approach. We have modified an existing bistable circuit, that of phage λ. With this approach, we used powerful genetic selections to identify functional circuits and selected for variants with altered behavior. The λ circuit involves two antagonistic repressors, CI and Cro. We replaced λ Cro with a module that included Lac repressor and several lac operators. Using a combinatorial approach, we isolated variants with different types of regulatory behavior. Several resembled wild-type λ—they could grow lytically, could form highly stable lysogens, and carried out prophage induction. Another variant could form stable lysogens in the presence of a ligand for Lac repressor but switched to the lytic state when the ligand was removed. Several isolates evolved toward a desired behavior under selective pressure. These results strongly support the idea that complex circuits can arise during the course of evolution by a combination of simpler regulatory modules. They also underscore the advantages of modifying a natural circuit as an approach to understanding circuit design, systems behavior, and circuit evolution.

[Keywords: Gene regulatory circuit; lambda phage; circuit design; Lac repressor; evolution of gene regulatory circuitry; systems behavior]

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Complex gene regulatory circuits involve interactions among many components, including specific DNA-binding proteins and the cis-acting sites to which they bind. Work over the past decades has led to a solid understanding of the operations of many important biological circuits and switches, including mechanistic detail and qualitative circuit diagrams of the connections [Wall et al. 2004]. This work has generally taken place in a reductionist framework, focusing ever more finely on the mechanistic details by which individual components of the circuit operate and interact.

At the same time, this work has revealed that many natural circuits involve factors such as nonlinearity and feedback that complicate their behavior and make it difficult to predict. For instance, in phage λ, the system we use here, nonlinearity in repressor binding to DNA arises from weak dimerization by CI and Cro and from cooperative DNA binding by CI repressor; expression of cl is also subject to both positive and negative feedback loops [Ptashne 1992, 2004]. The field of systems biology is concerned in part with developing approaches to understanding and predicting the “emergent” behavior of systems—behavior that results from factors like nonlinearity and feedback—using a combination of computational and experimental approaches [Kitano 2002]. Such endeavors ideally will complement the reductionist approach; together, they should provide far more complete descriptions of a system as a whole than either approach could do alone.

One approach to these questions is systematic experimentation on natural circuits, focusing on questions of systems behavior. In an early example of this, the chemotaxis system of Escherichia coli was found to be highly robust to changes in certain parameters—that is, its behavior does not change very much relative to the magnitude of the change in parameter [Alon et al. 1999]. With λ, we have found that the qualitative operation of the phage λ circuitry is robust to changes that affected the affinities of two repressors for their operators [Little et al. 1999]. More recently, we have characterized mutants that alter the set point of the threshold behavior in prophage induction [J.W. Little, unpubl.]. Although clearly feasible, however, this general approach has not yet enjoyed wide use.

A contrasting experimental approach to understanding emergent behavior is that of de novo design of gene regulatory circuits, using well-characterized components [Elowitz and Leibler 2000; Gardner et al. 2000; Guet et al. 2002]. In a relatively simple example of this approach,
the “toggle switch” (Gardner et al. 2000; see also Atkinson et al. 2003), two repressors whose activities could each be modulated by the experimenter were arranged such that each repressed the other. When one repressor was inactivated, the other came to predominate and continued to do so even under conditions that allowed the first repressor to be active if it was present. The converse was also true. Hence, the system was “ bistable”, two alternative stable states could exist. Significantly, however, in addition to several successful examples of this design, it was found that many other combinations were not bistable, in that one repressor could take over from the other when allowed to function. In these cases, the two regulatory states were not in balance with one another; one state was too “strong” for the other to be maintained.

In the present work, we have devised an alternative approach to systems design that combines the simplicity of de novo design with the interesting complexity shown by a natural circuit. We have taken a well-characterized natural circuit, the bistable switch of phage λ, and replaced one of the critical regulatory proteins, Cro repressor, with another well-studied regulatory protein, Lac repressor. We also included cis-acting sites for Lac repressor to allow it to execute the functions of Cro.

λ offers several advantages for this “hybrid” approach. First, as detailed following, the λ circuitry exhibits a range of interesting regulatory behavior (Pitashne 1992, 2004). Second, the powerful genetics available in the λ system allows us to identify functional circuits, select rare variants with desired characteristics, and evolve the behavior of these variants. Finally, the great depth of knowledge about λ allows interpretation at the mechanistic level.

The λ circuit can exist in two alternative regulatory states, and it can switch from one state to the other under control of the experimenter [Pitashne 1992, 2004]. The circuitry also involves an initial choice between the two alternative states, a choice made soon after a cell is infected and later stabilized by well-known regulatory interactions. A cell infected with λ can follow either of two exclusive pathways. It can follow the lytic pathway, in which the viral DNA replicates and is packaged into ~100 new virions, which are then released by cell lysis. Alternatively, the infected cell can follow the lysogenic pathway, leading to a stable association with the host; the phage DNA is integrated into the host genome and its lytic genes are repressed by CI repressor. In addition, although the lysogenic state is highly stable, it can switch to the lytic state in a “genetic switch” called prophage induction. When the host SOS system is induced by DNA damage [such as from ultraviolet [UV] irradiation], RecA protein becomes activated to a form that can mediate specific cleavage of CI, inactivating it and leading to expression of lytic genes and the lytic pathway [Little and Mount 1982; Roberts and Devoret 1983].

At the mechanistic level, the choice between lysis and lysogeny after infection is controlled mainly by the levels of the viral CII protein. CII activates the Prm promoter, leading to high levels of cl expression and favoring lysogeny. CII levels are controlled by the physiologic state of the cell, and by the level of another viral protein, CIII [Herskovitz and Hagen 1980]. Once a regulatory state has been chosen, it is maintained by the actions of two proteins, CI and Cro, acting mostly at a complex regulatory region termed the OR region [Fig. 1]. Both proteins bind to three sites in OR [Fig. 1B] but with differing affinities and different consequences [Ptashne 1992]. Each protein maintains its own expression while blocking that of the other. If CI predominates, it represses the lytic promoter Pr and activates the lysogenic promoter Prm, stabilizing the lysogenic state; it is generally believed that if Cro predominates, it stabilizes the lytic state by repressing Prm.

In addition to testing the feasibility of our approach, we had several specific goals in the present work. The first was to test whether a natural gene regulatory circuit retains a modular organization [Hartwell et al. 1999; Wolf and Arkin 2003]. Success would provide support for the idea that circuits could arise initially in evolution by proper combinations of functional modules. Second, it should allow us to explore issues of balance. We expected that only certain circuits would be in balance well enough to approximate the behavior of natural λ. Moreover, the use of a repressor whose activity we could manipulate offered the possibility of affecting the balance at will. Third, the use of genetics might allow evolution of circuits with novel behavior. We describe experiments that have met all of these goals.

Results

Approach

We chose to replace λ Cro rather than CI, because Cro is a much simpler protein than CI. CI has several features for which it would be difficult to substitute. In addition to its repressor activity, CI acts as an activator, stimulating its own expression from Prm about 10-fold. It also binds cooperatively to two adjacent operators, OR1 and OR2, and also has higher-order cooperativity, mediating looping between the OR and O2 regions. In contrast, Cro is only known to act as a repressor, and has little or no cooperative DNA binding.

Cro partially represses the early lytic promoters Pr and Prm, particularly at a late stage of the lytic cycle; failure of this function leads to overproduction of toxic gene products and to other poorly understood effects that interfere with lytic growth [Sergueev et al. 2001]. As a consequence, cro mutations are almost lethal; λ cro forms tiny plaques. In addition, it is believed that Cro operates during the switch from the lysogenic state to the lytic state, binding to OR3, thereby repressing Prm and making the switch irreversible. Accordingly, in replacing Cro we needed to provide the ability to repress Prm and Pr, and we also provided in some constructs the ability to repress Prm.

We needed to provide cis-acting sites at which another repressor with different DNA-binding specificity could
carry out the functions of Cro in the λ life cycle. An additional consideration in designing our system was that, as described earlier, both Cro and CI bind to the same sites, although with different affinities. This meant that we could not remove the Cro-binding sites without also affecting the functions of CI.

We chose Lac repressor to replace Cro for several reasons (Gilbert and Müller-Hill 1966). First, it has been extensively characterized. Second, its function can be modulated by adding isopropyl-β-D-galactopyranoside (IPTG) to the growth medium. IPTG weakens the affinity of Lac repressor for its operator (Riggs et al. 1970). Importantly, titration with IPTG should allow us to shift the balance of the circuitry. Finally, a feature of the λ lac operon that is fortunate for our purposes is that the lac operator lies after the start point of transcription, rather than being within the promoter as in most repressors. Hence, we could install lac operators at PL and PR without at the same time removing the binding sites for Cro and CI (Fig. 1).

Lac repressor does differ from Cro in at least two ways that are likely to bear on the behavior of the λ circuitry. First, Cro dimerizes weakly, providing a source of nonlinearity, whereas oligomers of Lac repressor are highly stable. Cro does not form higher-order oligomers. Second, in contrast, Lac repressor is a tetramer; moreover, it is highly cooperative binding, providing another source of nonlinearity.

As described in the introduction, for a circuit to be bistable, the two stable states must be in balance, such that one cannot readily take over once the other has been established. This balance can in principle be affected by the values of parameters such as the amount of a regulatory protein or its affinity for its operators. Because it is difficult to predict the proper choices of parameters to balance a new circuit, we tested many combinations using a combinatorial approach (Guet et al. 2002; Yokoyama et al. 2002), in which each of four cis-acting sites had several alleles with different properties. These sites included the lacO sites at PL and PR, at which either the native λ O3 site or a lacO was allowed; and the Shine-Dalgarno sequence in the message for Lac repressor (see Fig. 1D, Supplementary Fig. S1, Supplementary Table S1). At lacO sites, we provided the wild-type operator or one of four lacO mutants that weakened

![Figure 1. Design of λacI. A] Logical representation of the phage λ lys–lysogeny circuit. Plus (+) and minus (−) signs indicate activation and repression of promoter, respectively [Lac repressor could repress P RM when a lacO variant was in the position of O3]. CI and CII [not shown] are expressed from P L and P R, respectively. Expression of P L is controlled in λ by CI binding to two operators, O1 and O2, not shown here, which lie to the right of P L in positions analogous to O3 and O2, respectively. B] Control of regulatory state by the pattern of occupancy by CI or Cro. The circuit is maintained by the actions of CI and Cro at the OR region. CI stabilizes the lysogenic state, and Cro stabilizes the lytic state. C] Design of λlacI. Cro was replaced with lacI. LacO alleles were installed in two positions [PL and PR], at the position of O3, some variants had a lacO allele, and some had O3. The set of lacO alleles was used at all three sites. D] LacO alleles and the Shine-Dalgarno sequences used in combinatorial approach. Allele A is wild-type lacO; mutations in other lacO alleles are in bold type; binding is progressively weaker going from B to E (Betz et al. 1986). W is λ O3. In box SD, Shine-Dalgarno sequences and start codons are in bold type; SD alleles allow progressively less-efficient translation going from A to F (Gardner et al. 2000). E] O3 and lacO are in bold type. O3 and O2 are italicized. Locations of −35 and −10 regions for the promoters are shown. The base substitutions at the ends of the lac at O3 should not affect LacI binding (Betz et al. 1986).
binding to varying extents; the same five lacO alleles were possible at each of the three sites. The spacing between lacO located at $O_R^3$ and $P_R$, 72 bp, did not support highly efficient looping (Müller et al. 1996). At the Shine-Dalgarno sequence, we used a series of sequences that show a wide range of translation efficiencies in a different context [Gardner et al. 2000]; we have not tested directly their efficiencies in the present context.

**Isolation of lacI variants**

Pools of variant $O_R$ regions were cloned into a new $\lambda$ vector that allows cloning of the $O_R$ region [Michałowski et al. 2004]. Regulation in this vector, $\lambda$JL351, differs somewhat from that of wild-type in two respects. It contains a silent XhoI site early in $cl$, which weakens by about half the strength of $P_{RM}$ (probably by reducing translation efficiency of the leaderless $P_{RM}$ message), presumably leading to lower levels of CI. Probably as a consequence of this, lysogens of $\lambda$JL351 are induced by lower levels of UV light than is wild-type $\lambda$. This phage carries a kanamycin marker, allowing us to select for the ability to form stable lysogens. We cloned the variant $O_R$ regions, plus the lacI gene in place of cro [see Supplementary Material] using as vector pooled DNA from five variants with different lacO alleles at $P_L$. Ligation mixtures were packaged, resulting in a pool of phage [Supplementary Table S2].

We then applied an initial genetic selection for the capability to grow lytically. The pool was plated for plaques in the absence of IPTG or at various levels of capability to grow lytically. The pool was plated for

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**Lytic growth of all isolates was sensitive to the presence or absence of IPTG** [Table 1; Supplementary Table S3]. Many variants could establish and maintain the lysogenic state [Table 1; Supplementary Table S3]. Finally, we tested whether lysogenizing variants could carry out prophage induction [Fig. 2; Supplementary Table S3]. For this process to occur, CI levels in the lysogen must be reasonably close to those of wild type, and the lytic state must be able to take over when CI is removed. We found that several variants could execute this switch [Fig. 2]; the behavior of AWCF was closest to that of wild type.

From these data we conclude, first, that it is possible to isolate lacI variants with regulatory and phenotypic behavior similar to those of wild-type $\lambda$. Importantly, these findings validate our approach of replacing one crucial regulatory protein with another. Second, we conclude that many variants are defective in one or more aspects of $\lambda$ behavior, confirming the utility of a combinatorial approach. We next discuss which combinations are acceptable, then turn to a more detailed analysis of selected variants.

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**Figure 2.** UV dose responses for prophage induction. Exponentially growing cultures of lysogens were centrifuged, suspended in TMG, and irradiated (see Materials and Methods) at the indicated doses; aliquots were diluted 10-fold in LBGM and shaken for 2 h at 37 °C in the dark, treated with CHCl$_3$ and titrated. [Crosses] Wild type; [diamonds] AWCF; [stars] AWBF; [squares] AWCE; [triangles] AWBE.

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**Table 1. Behavior of selected lacI variants**

| [IPTG] | 0 | 10$^{-4}$ M | 10$^{-3}$ M | 10$^{-3}$ M | 0 |
|-------|---|-------------|-------------|-------------|---|
| Name | MitC$^b$ | No | No | No | No |
| WT | L | L | L | L | S |
| ABDA | M | M | No | No | S & U |
| ADDD | M | M | No | S | U |
| AEEF | No | No | S | No | No |
| AEDA | M | M | No | No | U |
| AWBF | M | M | No | No | S |
| AWCF | L | L | No | L | S |
| AWDF | S | M | No | M | S |
| CECA | S | No | No | No | No |
| CWCMD | M | No | No | No | S |

Variants were symbolized by the alleles they carry at each of the four variable sites [$P_L$-$O_R$-$P_R$-$SD$; see Fig. 1 and text].

$^{a}$ No$^{a}$ indicates no plaque formation; “S”, “M”, or “L” indicates the size of plaques, small, medium, or large, respectively.

$^{b}$ MitC$^b$ indicates presence of 1.0 µg/mL mitomycin C in top and bottom agar.

$^{c}$ “S”, “U”, or “No” indicates stable, unstable, or no lysogen, respectively, in the absence of IPTG; “unstable” means that colonies of lysogens were of heterogeneous size and that they did not grow to high density on inoculation into liquid media.
Acceptable combinations of cis-acting sites

It is difficult to infer general rules for acceptable combinations of cis-acting sites [Table 1; Supplementary Table S3]. At $P_\text{R}$, the strongest $lacO$ allele, “A”, was preferred [28 of 33]. The $P_\text{R}$ operon encodes several proteins that are toxic when overproduced [Sergueev et al. 2001]. This pattern suggests that repression of $P_\text{R}$ is the most important function for Lac repressor during lytic growth (see also following). At $O_\text{R}3$, both the native $O_\text{R}3$ site and $lacO$ variants were allowed for lytic growth. However, many variants with a $lacO$ at this site could not lysogenize, even in the presence of IPTG. We do not fully understand the mechanistic basis for this pattern. Although Lac repressor might block $P_\text{RM}$ by binding to the $lacO$ site, it should not bind tightly in the presence of IPTG (see also Discussion). It is possible that the substitutions between the −10 and −35 regions of $P_\text{RM}$ alter the properties of this promoter. At $P_\text{R}$, all five $lacO$s were found, but “A” was rare; presumably this would cause too low a level of $P_\text{R}$ expression. In the $lacI$ SD sequence, none of the variants with “W” in $O_\text{R}3$ had the strongest SD “A”; in contrast, half of those with $lacO$ alleles at $O_\text{R}3$ had “A” in SD. A cluster of variants (AWBE to AWEE; Supplementary Table S3) had roughly similar behavior. This group had “AW” in the first two positions and a relatively weak SD sequence at $lacI$; none had the strongest $lacO$ (A) at $P_\text{R}$. These did differ from one another, mostly in the phage yield after prophage induction, but all gave good lytic growth and had the ability to lysogenize, indicating that there is a range of parameters for which the $\lambda lacI$ system can maintain a stable regulatory state and switch to an alternative state.

Balance in the $\lambda lacI$ system

The properties of many variants gave insight into the important systems property of balance. We found that the balance of the $\lambda$ system could be perturbed in our isolates by adding or removing IPTG, which weakens specific DNA binding by Lac repressor. Balance could be affected in each of two important aspects of specific DNA binding by Lac repressor. Balance could be isolated by adding or removing IPTG, which weakens properties of this promoter. At PR, all five $lacO$s were found, but “A” was rare; presumably this would cause too low a level of $P_\text{R}$ expression. In the $lacI$ SD sequence, none of the variants with “W” in $O_\text{R}3$ had the strongest SD “A”; in contrast, half of those with $lacO$ alleles at $O_\text{R}3$ had “A” in SD.

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Lytic growth of almost all variants was responsive to IPTG. All but three isolates could not form plaques in the presence of $10^{-4}$ M IPTG, where the activity of Lac repressor leads to overproduction of toxic genes or the infected cell might usually follow the lysogenic pathway. Cells might be unable to complete the lytic pathway because weak repression by Lac repressor leads to overexpression of toxic phage functions, such as $\text{kl}$, during lytic growth [Sergueev et al. 2001]. This is likely for variants that could not form stable lysogens. Conversely, variants that could form stable lysogens might usually or always choose the lysogenic pathway after infection. That is, IPTG would upset the balance between these two choices.

We tested these possibilities by assaying plaque formation in the presence of Mitomycin C, an SOS-inducing agent that leads to cleavage of CI and thereby blocks the lysogenic pathway. We expected that a variant that was capable of lytic growth but usually followed the lysogenic pathway would form a plaque under these conditions. Among variants unable to form stable lysogens, 9/12 did not form plaques in the presence of Mitomycin C and $10^{-4}$ M IPTG [Supplementary Table S3], suggesting that these could not complete the lytic cycle. Among stably lysogenizing variants, 10 could also not form plaques under these conditions. In contrast, seven others could do so, suggesting that in these cases IPTG conferred a bias toward the lysogenic response. A direct test of lysogenization frequency after single infection in the absence or presence of IPTG confirmed this prediction for two isolates (ADDD and AWCF; Table 2), and showed that the choice was also biased by IPTG in two variants (ABDA and AWBF) that could not complete the lytic pathway. At the mechanistic level, we surmise that poor repression by Lac repressor led to overproduction of $\text{CII}$ and $\text{CIII}$ and expression of $P\text{RE}$ [Herskowitz and Hagen 1980].

In another case, removal of IPTG also upset the balance, but in this case it disrupted the balance between two stable states of the system. The CECA variant formed lysogens at $10^{-4}$ M IPTG, and these were almost as stable at $10^{-4}$ M IPTG as those of wild-type $\lambda$; it is much more efficient after multiple infection. Failure of plaque formation might also result from one or both of two causes—there might be overexpression of toxic genes or the infected cell might usually follow the lysogenic pathway. Cells might be unable to complete the lytic pathway because weak repression by Lac repressor leads to overexpression of toxic phage functions, such as $\text{kl}$, during lytic growth [Sergueev et al. 2001]. This is likely for variants that could not form stable lysogens. Conversely, variants that could form stable lysogens might usually or always choose the lysogenic pathway after infection. That is, IPTG would upset the balance between these two choices.

Table 2. Effect of IPTG on lysogenization frequency

|         | −IPTG | +IPTG |
|---------|-------|-------|
| WT      | 1.2   | 1.2   |
| ADDD    | 2.3   | 57    |
| AWCF    | 0.9   | 38    |
| ABDA    | 0.2   | 18    |
| AWBF    | 0.3   | 26    |

Each number shows lysogen efficiency (%). Cells were grown, singly infected, and plated on kanamycin plates with or without $10^{-4}$ M IPTG [see Materials and Methods]. Lysogenization by wild-type $\lambda$ after single infection is very inefficient [Kourilsky 1973]; it is much more efficient after multiple infection.
Evolution of \( \lambda \)acl variants

We tested whether variants could evolve under selective pressure. Because most had defects in at least one aspect of \( \lambda \) regulatory circuitry, we could select for secondary variants with altered phenotypes. Two examples follow. First, \( \lambda acl \) variants whose \( lacO \) allele at \( P_L \) was “C” could not make plaques in the presence of \( 10^{-5} \) M IPTG. We isolated two independent variants of CWCD that could do so. Both contained mutations in \( P_L \): \(-10\) GATACT to GATGCT, or \(-35\) TTGACA to TTGATA

that probably weakened \( P_L \). We infer that the growth defect of CWCD with \( 10^{-5} \) M IPTG resulted from weak repression of \( P_L \), giving overexpression of toxic gene products. Second, we identified many pairs of variants in which a single site change of \( lacO \) or SD caused an altered phenotype [Supplementary Table S4]. For example, AECF and AECF differed only in the \( lacO \) sites in \( P_R \): AECF could form plaques without IPTG, but AEAE could not. Three independent secondary variants of AEAE that could do so were isolated. All three had changes in \( lacO \) in \( P_R \). The changes probably weakened the affinity of Lac repressor for this site, implying that the growth defect of AEAE results from too-strong repression of \( P_R \), which restricted early lytic gene expression more than in wild type.

Discussion

Our success at replacing Cro with Lac repressor in the context of the intact phage circuitry has numerous implications for regulatory mechanisms, systems properties, and evolution of gene regulatory circuitry, as we discuss following. Our work also illustrates the advantages of a genetic approach to analyzing circuit design and systems behavior. The use of a combinatorial approach allowed us to identify acceptable circuits from a broad range of possibilities; combinatorial approaches have been similarly used in de novo design to identify toggle switches that are in balance and circuits with a variety of connectivities.

Mechanisms

In the Results, we discussed several examples in which the behavior of variants, or comparisons between pairs, appear to be consistent with current understanding of \( \lambda \) circuitry and the properties of Lac repressor. Conversely, there are many cases in which we do not understand the behavior. A major advantage of using \( \lambda \) and Lac repressor is that our depth of knowledge helps us to recognize the limits to current understanding and departures from it.

For instance, we found that variants ACDF and CDBA were able to form stable lysogens, despite the fact that both have a 1-bp deletion in \( O_{R1} \). This mutation would probably weaken the strength of \( P_R \), but should completely abolish binding of CI to this site. It is unclear how the lysogenic state could be maintained in such phage.

With Lac repressor, our results highlight a limitation in our current understanding. Careful studies have not been done to assess the interaction of Lac repressor with mutant operators as a function of IPTG concentration. Accordingly, we do not know how various levels of IPTG affect these interactions.

An additional complication in the case of our constructs is that Lac repressor probably supports looping between \( lacO \) sites at \( P_L \) and \( P_R \); in addition, in phages with a \( lacO \) site at \( O_{R3} \), three different loops are possible. Looping between the sites at \( O_{R3} \) and at \( P_R \) may be
weaker than the long-range looping between either site and that at \( P_R \). In any case, in vitro studies on supercoiled DNA have shown that looped complexes with DNAs containing two operator sites are far more stable than complexes with DNA as a single operator, presumably because of the local concentration effects and juxtaposition due to supercoiling [Hsieh et al. 1987; Whitson et al. 1987; Vologodskii et al. 1992]. Dissociation at each of the individual operator sites may mirror release of LacI from a single-operator DNA, but the high local concentration of a second high-affinity site would favor reformation of the looped structure and ensure a long half-life for the protein–DNA complex.

The consequences of looping and the uncertain energetics for all of the possible looped forms make it difficult to predict in detail the behavior of \( \lambda lac \) phages. However, we suggest that looping could account for the finding that most phages with a \( lacO \) at the position of \( O_R3 \) could not form stable lysogens, even with \( 10^{-4} \) M IPTG. Possibly looping increased the residual affinity of Lac repressor enough to allow substantial occupancy of this site, repressing \( P_{RM} \) and preventing maintenance of lysogeny.

Another unexpected finding was that the AACA variant showed a “reverse” response to IPTG—it could lytically grow if some transcripts from \( P_R2 \) were repressed for several minutes, an effect impossible in \( P_R \) because both of its regulatory states are stable. The lytic state does not need to be stable during normal \( \lambda \) growth, because it is transient. When lytic functions are blocked by mutation, however, the circuitry can exist in a stable “anti-immune” state in which Cro predominates [Eisen et al. 1970; Calef et al. 1971]. We have not shown that our \( lacI \) variants can adopt this anti-immune state. Hence, it is possible that the circuits studied here are not bistable in this sense.

It is becoming increasingly clear that many biological systems have a modular organization. Theoretical approaches suggest that modularity of circuits is needed for robustness and facilitating the generation of variation [Wolf and Arkin 2003]. Although definitions of a module vary widely, we use the term here to refer to a regulatory protein, the \( cis \)-acting sites to which it binds, and its actions while bound. A plausible model for creation of complex regulatory circuits is that they arise by random associations between functional modules, followed by selection for those with adaptive properties. However, it is possible in principle that during continued selection and refinement of a circuit, the modules would lose their identity by extensive interlocking with other elements of the system. Our ability to replace Cro with Lac repressor and its \( cis \)-acting sites provides a straightforward counterexample. Clearly the modularity of the \( \lambda \) circuit has been retained sufficiently to allow this substitution to take place.

On the basis of this work, we suggest a plausible pathway for creation of a complex regulatory circuit from simpler parts [Ptashne and Gann 1998]. A regulatory circuit like that in AWCF could arise by nonhomologous recombination between one module containing CI and its operators and another with Lac repressor and its operator at \( O_R \). This would require only that a promoter like \( P_R \) be present in the \( \lambda \) module. Because it is likely that looping occurs in our variants and strengthens the binding of Lac repressor, it would be helpful if another \( lacO \) site was nearby to allow looping. In this case, a module carrying two \( lacO \) sites and the \( lacI \) gene would undergo insertion of the \( P_R \cdot P_{RM} \cdot CI \) module between the two \( lacO \) sites, creating a functional circuit.

It is widely believed that changes in \( cis \)-acting sites more often underlie the evolution of morphological diversity than do changes in gene number or protein functions [Carroll 2000]. Our findings support this general model. We identified 38 pairs of variants that differed in only one \( cis \)-acting site [Supplementary Table S4]. Half of these pairs were in the group of variants including AWCF, and these usually had quantitative differences in phage yield. In most of the other pairs, the two variants exhibited distinctly different phenotypes. Moreover, in evolution experiments, almost all mutations were in the \( lacO \) and promoter regions, not in protein-coding regions, even though the target sizes of protein-coding regions are much larger than those of \( cis \)-acting sites. Continued analysis of these variants, and extensions of our approach, should provide further insight into ways that evolution of \( cis \)-acting sites modifies the behavior of gene regulatory circuits.

**Behavior and evolution of regulatory circuitry**

The likelihood that Lac repressor-mediated looping is taking place implies that the connectivity of the circuits studied here differ somewhat from that of normal \( \lambda \), because expression of \( P_L \) and \( P_R \) is likely to be controlled in parallel. The consequence of this for lytic growth is uncertain. The only essential protein expressed from \( P_L \) is the \( N \) protein. If \( P_L \) were repressed for several minutes, \( N \) levels would drop substantially, because this protein is very unstable, but that might have little effect on lytic growth if some transcripts from \( P_R \) had already been antiterminated by the action of \( N \) [Roberts 1993].

The \( \lambda \) regulatory circuitry is often termed “bistable,”
Materials and methods

Media

Tryptone broth and LB were as described [Miller 1972] and were supplemented with antibiotics as appropriate, except that kanamycin was at 10 µg/mL. LBGM and LBMM were LB supplemented with 1 mM MgSO₄ and 0.2% glucose or 0.2% maltose, respectively. TMG was 10 mM Tris-HCl at pH 8.0, 10 mM MgSO₄, and 10 µg/mL gelatin.

Phage and bacterial strains

Phage strain λL351 was used as wild type [Michalowski et al. 2004]. It differs slightly from wild-type λ in that its set point for UV induction is ~6–7 J/m² instead of 10–12 for wild type. It contains a unique Xhol site early in Cl. In λL351, the Tn5 kan marker, followed by the strong trp a terminator, replaced λ DNA from position 23901 to 26818 in the b2 region. Its phenotype differs slightly from wild type, in that it is somewhat easier to induce with UV. λL387 was λL351 with v1 and v3 mutations in O₂α2 and O₂α1, respectively, and was used as the cloning vector for the O₂ region [Michalowski et al. 2004] as described in the Supplemental Material. Derivatives of λL387 with various lacO alleles at P₂ were made by two sequential phage-by-plasmid crosses. First, λL387 was crossed with a plasmid that contained P₂ and the v2 mutation in O₂. Cross progeny were plated on strain JL5892 [JL2497 carrying pLR1 [Whipple et al. 1994], which provides a low level of CI). On this mixed indicator, and carrying a plasmid, pA3B2 [Whipple et al. 1994], providing CI. In \( \text{lacO} \), we plated phages on a 4:1 mixture of JL2497 and JL387, which could plate. The resulting phage (λL1000) formed small clear plaques; presence of v2 was verified by DNA sequencing. Second, λL1000 was crossed with each of five plasmids carrying \( \text{P}_1 \) and one of five lacO alleles. To distinguish recombinants carrying lacO in the \( \text{P}_1 \) region from λL1000, we plated phages on a 4:1 mixture of JL2497 and JL895 [a derivative of JL2497 with \( \text{F}^- \), Tn3 replacing \( \text{F}^- \). TMG, and carrying a plasmid, pA3B2 [Whipple et al. 1994], providing a low level of CI]. On this mixed indicator, λL1000 formed clear plaques, whereas v2 recombinants formed turbid plaques. Presence of the lacO site was verified by DNA sequencing. DNA was made from each of five recombinants, using the QIagen lambda DNA kit; equal amounts of DNA from each recombinant were pooled to provide the vector DNA. From this pooled DNA, left and right arms were purified as described (Michalowski et al. 2004), for the right arm; in addition, the left arm preparation was treated a second time with streptavidin beads, as described, to recover right arms, which were pooled with the right-arm preparation. Left arms were treated with shrimp alkaline phosphatase after right arms were removed for the second time.

Bacterial strain JL6142 \( \Delta [\text{lacIPOZYA}] \) was derived in two steps from strain JL2497 [Little et al. 1999]. First, a derivative lacking the \( \text{F}^- \). Tn9 was isolated by growth in the absence of chloramphenicol and screening among chloramphenicol-sensitive colonies for those not sensitive to M13. Second, \( \text{lacI} \) and the \( \text{lac} \) operon were deleted as described [Datsenko and Wanner 2000]. Strain JL7223 was an \( \text{hflA150} \) mutant of JL6142, and JL7223 was a derivative of JL6142 as donor, selecting for tetracycline resistance and screening among chloramphenicol-sensitive colonies for those not sensitive to M13. Second, JL6142 was crossed with a plasmid that lacked the \( \text{F} \). Tn5 marker, followed by the strong \( \text{trp} \) a terminator, replaced λ DNA from position 23901 to 26818 in the b2 region. Its phenotype differs slightly from wild type, in that it is somewhat easier to induce with UV. JL387 was JL351 with v1 and v3 mutations in O₂α2 and O₂α1, respectively, and was used as the cloning vector for the O₂ region [Michalowski et al. 2004] as described in the Supplemental Material. Derivatives of JL387 with various lacO alleles at P₂ were made by two sequential phage-by-plasmid crosses. First, JL387 was crossed with a plasmid that contained P₂ and the v2 mutation in O₂. Cross progeny were plated on strain JL5892 [JL2497 carrying pLR1 [Whipple et al. 1994], which provides a moderate level of CI], on which λvir \( \text{v1 v2 v3} \), but not JL387, could plate. The resulting phage (JL1000) formed small clear plaques; presence of v2 was verified by DNA sequencing. Second, JL1000 was crossed with each of five plasmids carrying P₂ and one of five lacO alleles. To distinguish recombinants carrying lacO in the \( \text{P}_1 \) region from JL1000, we plated phages on a 4:1 mixture of JL2497 and JL895 [a derivative of JL2497 with \( \text{F}^- \). Tn3 replacing \( \text{F}^- \), TMG, and carrying a plasmid, pA3B2 [Whipple et al. 1994], providing a low level of CI]. On this mixed indicator, JL1000 formed clear plaques, whereas v2 recombinants formed turbid plaques. Presence of the lacO site was verified by DNA sequencing. DNA was made from each of five recombinants, using the QIagen lambda DNA kit; equal amounts of DNA from each recombinant were pooled to provide the vector DNA. From this pooled DNA, left and right arms were purified as described [Michalowski et al. 2004], except that we used NsiI, which cuts just distal to cro, for the right arm, in addition, the left arm preparation was treated a second time with streptavidin beads, as described, to recover right arms, which were pooled with the right-arm preparation. Left arms were treated with shrimp alkaline phosphatase after right arms were removed for the second time.

Screening of λ lacI

Phages of this library [made as described in Supplemental Material] were plated on JL6142 on tryptone plates without or with IPTG at 10⁻⁵ M or 10⁻⁴ M. Turbid or small plaques were purified and analyzed by PCR; isolates with a fragment of the expected size were further characterized. Segments of the λlacI were amplified by PCR of plaques. PCR products (\( \text{P}_1 \)–O₂ and CI–P₁₋₂₆₈₁₈–NsiI) were sequenced by automated cycle sequencing at the Division of Biotechnology, University of Arizona. To test whether recombinants existed that formed clear plaques, we analyzed 20 clear plaques by PCR, and none had an insert; an additional 200 clear plaques were tested on a mixture of JL2497 and JL5434 [Michalowski et al. 2004], a mixture on which recombinants and vector form turbid and clear plaques, respectively; all isolates formed clear plaques, suggesting that recombinants forming clear plaques on JL6142, if present, were very rare.

UV dose responses for prophage induction

This was carried out as described [Michalowski et al. 2004]. Stably lysogenizing variants were first screened at a dose of 40 J/m² [Supplementary Table S3]. For selected variants, single lysogens were identified by a PCR test [Powell et al. 1994] and were further analyzed by irradiating with various doses at 0.5 J/m²/sec [Fig. 2].

Lysogenization tests

To test for ability to lysogenize, we centrifuged JL6142 grown in LBMM to 2 × 10⁹/mL, concentrated it 10-fold in TMG, and mixed it with phage at an m.o.i of ~5 [a condition that favors the lysogenic pathway], after 10 min at room temperature, aliquots were diluted into LBGM containing 0, 10⁻⁵, or 10⁻⁴ M IPTG, shaken 30 min at 37°C, and spread onto with tryptone plates containing 10 µg/mL kanamycin and the corresponding amount of IPTG [results given in Table 1; Supplementary Table S3]. To test for lysogenization frequency after single infection [Table 2], we grew cells as described earlier, infected them at a multiplicity of 0.01, diluted and incubated them as described earlier, and plated them in top agar with or without IPTG in top and bottom agar as indicated.

Isolation of secondary variants with altered phenotypes

Multiple plate stocks of λlacI variants were made on JL6142 from single plaques, using fresh tryptone plates and 6 mL of 0.35% top agar. Stocks were plated, and variants of particular phenotypes were chosen. Segments of these variants were amplified by PCR of plaques. PCR products (\( \text{P}_1 \)–O₂ and CI–P₁₋₂₆₈₁₈–NsiI) were sequenced.

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