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A synthetic phage λ regulatory circuit

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Analysis of synthetic gene regulatory circuits can provide insight into circuit behavior and evolution. An alternative approach is to modify a naturally occurring circuit, by using genetic methods to select functional circuits and evolve their properties. We have applied this approach to the circuitry of phage λ. This phage grows lytically, forms stable lysogens, and can switch from this regulatory state to lytic growth. Genetic selections are available for each behavior. We previously replaced λ Cro in the intact phage with a module including Lac repressor, whose function is tunable with small molecules, and several cis-acting sites. Here, we have in addition replaced λ CI repressor with another tunable module, Tet repressor and several cis-acting sites. Tet repressor lacks several important properties of CI, including positive autoregulation and cooperative DNA binding. Using a combinatorial approach, we isolated phage variants with behavior similar to that of WT λ. These variants grew lytically and formed stable lysogens. Lysogens underwent prophage induction upon addition of a ligand that weakens binding by the Tet repressor. Strikingly, however, addition of a ligand that weakens binding by Lac repressor also induced lysogens. This finding indicates that Lac repressor was present in the lysogens and was necessary for stable lysogeny. Therefore, these isolates had an altered wiring diagram from that of λ. We speculate that this complexity is needed to compensate for the missing features. Our method is generally useful for making customized gene regulatory circuits whose activity is regulated by small molecules or protein cofactors.

circuit design | epigenetic switch | gene regulation | systems biology

Living cells contain a wide variety of gene regulatory circuits, with widely varying degrees of complexity. How did complex circuits arise? It is plausible that they arose from simpler ones during the course of evolution. We have suggested (1–3) that this process of gaining complexity can involve at least two related types of changes. First, qualitatively new features can be added to a preexisting simpler circuit. These features can include new proteins, new cis-acting sites, or new interactions that alter the wiring diagram of the circuit. Addition of new interactions can lead to novel systems properties such as nonlinearity and feedback, which confer advantageous systems behavior such as stability (4), bistability (5, 6), and robustness (7–9) upon a circuit. Changes in the wiring diagram, particularly in the arrangements of cis-acting sites, can also give rise to diversity during speciation (10). A second type of change is refinement of system parameters such as promoter strength or the affinity of DNA-binding proteins for their sites, improving fitness. However, contemporary circuits retain few vestiges of their origins that allow us to trace evolutionary pathways. Thus, simple experimental systems are needed to understand how simple circuits can be elaborated to create more complex ones.

One approach is to develop synthetic circuits from well characterized components. This approach can give rise to circuits with biologically relevant behaviors, such as bistability (5), and novel circuits with different wiring diagrams (11) whose properties can then be explored. An alternative approach, which we take here, is to remove features from an existing circuit, both to simplify its operation and to test which features are essential for proper operation. This approach partially uncouples the circuit but uses a functional system with behaviors resembling those of the natural system. In principle, this approach can also provide simplified circuits that can then evolve more complex circuitry and behavior.

In this work, we have applied this approach to a well characterized regulatory circuit, that of phage λ. λ has several advantages for this approach. First, λ is well understood at the mechanistic level, facilitating interpretation of circuit behavior. Second, the λ circuit exhibits several regulatory decisions, stable states, and switches (12). Finally, we can select genetically for functional circuits and rare variants, allowing evolution of circuit behavior.

The λ gene regulatory circuit is bistable. A bistable gene regulatory circuit can adopt two alternative stable states. In λ, these states are the lytic and lysogenic states. In addition, λ can switch from the lysogenic state to the lytic state (12). Although the lytic state is ordinarily short-lived because of cell lysis, a cell can persist as an "anti-immune" state when lytic functions are blocked by mutation (13, 14). Well characterized mechanisms stabilize the two regulatory states, operating primarily by the action of two regulatory proteins, CI and Cro, in a complex 100-bp region termed the O_R region (Fig. 1*A*).

A bistable circuit must possess at least three features (6). The first is balance between the two states of the system. That is, forces stabilizing each state must be of comparable strength; if one state is too strong, a system starting in the weaker state will switch to the stronger. Imbalance was observed, for instance, in the synthetic toggle switch (5), for which certain constructs were not in balance, and this behavior could be rationalized at the mechanistic level. Balance is particularly important in several aspects of the λ circuit. The stability of the lysogenic state must be high enough to allow lysogens to persist, but not so high that they cannot switch to the lytic state. In addition, the two pathways of the lysis-lysogeny decision must be balanced so that both outcomes can occur after infection.

Second, a bistable circuit must contain some type of nonlinearity, that is, a response that is greater than proportional to the stimulus. In the λ circuit, nonlinearity is provided by relatively weak dimerization of CI and Cro, and by several forms of cooperative DNA binding by CI. The third required feature for bistability is positive feedback or double-negative feedback. In the λ circuit, Cro and CI repress one another's expression, providing double-negative feedback, and CI positively autoregulates its own expression, affording positive feedback.

As stated, the lytic state does not ordinarily persist; presumably the λ circuit is not under selective pressure for a stable anti-immune state. Instead, we surmise that the features that make the λ circuit bistable are present both to allow one stable

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Abbreviations: TetR, Tet repressor; aTc, anhydrotetracycline; SD, Shine–Dalgarno; IPTG, isopropyl β-D-thiogalactoside; TL phage, Tet-Lac phage.

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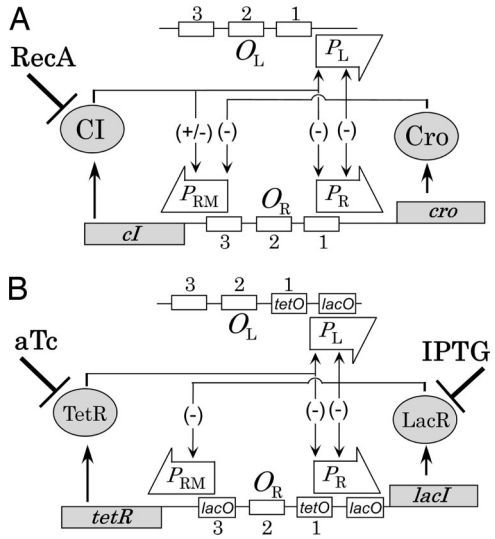

Fig. 1. Design of TL phage. (*A*) Representation of the phage λ regulatory circuit. (+) and (−), activation and repression of a promoter, respectively. CIII and CII (not depicted) are expressed from P_L and P_R, respectively. P_L is repressed by CI binding to O_L1 and O_L2. (*B*) Design of TL phage. The *cI* and *cro* genes were replaced with *tetR* and *lacI*, respectively. Alleles of *lacO* were installed in three positions (P_L, O_R3, and P_R), and *tetO* alleles were installed at two positions (O_L1 and O_R1).

state (the lysogenic state) and to help create an optimal balance between this state and the lytic pathway. Accordingly, we have focused, here and in previous work, on attempting to simplify the λ circuitry while still retaining qualitatively normal phenotypic behavior. We find that several features of the λ circuit are individually dispensable by this criterion, including positive autoregulation of CI (3) and cooperative DNA binding by CI (A. C. Babić and J.W.L., unpublished results). In each case, however, the other feature remained, and it is possible (3) that these two functions are partially redundant in providing positive feedback, a form of redundancy we term "circuit-level redundancy." In the present work, we have extended this approach by removing both these features provided by CI.

In a previous study (2), we replaced Cro repressor with another well studied regulatory protein, Lac repressor (LacR), and included cis-acting sites for LacR to allow it to execute the functions of Cro. These results revealed that the λ gene regulatory circuit retains a modular organization and showed the effectiveness of the module-replacement approach. In that work, the nonlinearity provided by weak Cro dimerization was removed, but a possibly compensating source of nonlinearity was provided by cooperative binding of tetrameric LacR. In a later study (15) and in the present work, we used a mutant LacR that cannot form tetramers and therefore cannot mediate interactions between operators. Accordingly, this protein more closely resembles Cro protein, but the relatively weak dimerization of dimeric LacR (16) again provides a residual source of nonlinearity.

In this work, in addition to replacing Cro with LacR we have replaced λ CI with a module that included Tet repressor (TetR) and several *tet* operators, creating a family of λ Tet-Lac or TL phages. TetR has been extensively characterized (17, 18), and its function can be modulated by adding anhydrotetracycline (aTc) to the growth medium. aTc weakens the affinity of Tet repressor for its operator. Therefore, titration with aTc should allow us to shift the balance of the circuitry. Importantly, TetR is not known to activate transcription or bind cooperatively to DNA as CI does. Because of the design of our circuit (Fig. 1), negative autoregulation can occur by LacR but not by TetR. The circuit design should also allow double-negative feedback of TetR and LacR upon each other. At least one element of nonlinearity, the weak dimerization of LacR, also remains. Dimerization of Tet repressor has not been analyzed directly, so it is unclear whether this feature provides any nonlinearity.

In the circuit design used here (Fig. 1), as in our previous studies, the properties of several cis-acting sites are expected to affect the balance of the synthetic circuit. Because we cannot predict *a priori* the balance in the present circuit, we tested many possible arrangements of cis-acting sites using a combinatorial approach. We were able to isolate several TL phages with behavior similar to that of λ. Strikingly, however, we found that their circuitry has an altered wiring diagram.

Results

Design and Isolation of TL Phages. In previous work, we installed *lacO* sites to allow LacR to carry out the repressive functions of Cro at the P_{RM}, P_R, and P_L promoters. In λ, CI also affects expression of these three promoters. Repression of P_L and P_R in a lysogen is needed to prevent expression of the lytic genes and execution of the lytic pathway. Therefore, we put *tetO* sites at operators O_R1 and O_L1 to repress P_R and P_L with TetR, respectively [Fig. 1; see also supporting information (SI) Fig. 4]. We chose not to put *tetO* at P_{RM}, because most negative autoregulation of *c*I in λ involves CI-mediated looping, which TetR cannot support. Because TetR cannot support positive autoregulation we did not install a *tetO* site adjacent to P_{RM}. Finally, to make a higher level of TetR possible, we installed a 5′ leader with a Shine–Dalgarno (SD) sequence on the *tetR* mRNA; in contrast the *c*I mRNA lacks a 5′ leader (19).

To identify functional circuits we used a combinatorial approach, in which each of seven cis-acting sites had several alleles with different properties. These sites included the *lacO* sites at P_{RM} and P_R. At P_L, WT *lacO* was installed to reduce the complexity and provide strong repression, because this allele was found in circuits most closely resembling λ (2). Several alleles were also provided for *tetO* sites at O_R1 and O_L1 and for SD sequences in the messages for Lac and Tet repressors. At *lacO* and *tetO* sites, we provided the WT operator (termed A in each case) or one of two operator mutations (termed B and C) that weakened binding to progressively greater extents; the same three alleles were possible at each site. Six SD sequences with a range of efficiencies (5) were provided, termed A–F in order of decreasing efficiency. Finally, because TetR lacks the ability to stimulate transcription of *tetR*, we provided two alleles of P_{RM}, one with the WT −35 and −10 regions and one with an up-mutation (20) in the −35 region of the same construct. Each of the three λ promoters retained its −35 and −10 regions but most of the sequence between these regions was altered, possibly affecting promoter strength. In total, 5,832 different combinations should be possible. Combinatorial pools of phage were prepared as described in *SI Materials and Methods*.

We applied an initial genetic selection for the ability to grow lytically by plating the pool for plaques in the absence of isopropyl β-D-thiogalactoside (IPTG) or at 10^{-5} M IPTG, and screening for turbid plaques. Separately, we applied a selection for the ability to form lysogens in the absence of IPTG. Twenty-six turbid plaque-forming phages and 3 single or double lysogen-forming phages were isolated and characterized. Sequence analysis identified 26 different variants (SI Table 2); a subset of the isolates is described in Table 1. We term these isolates TL phages for brevity.

Formation of Stable Lysogens. Thirteen of 26 variants could form stable lysogens. Among lysogenizing variants, nine could form stable single lysogens (Table 1 and SI Table 3). The other four formed only lysogens with two or more prophages, presumably because repression of the lytic genes required a higher gene dosage of the regulatory proteins. All of the lysogenizing variants

Table 1. Sequence and behavior of selected TL phages

	Phage isolate											
TL no.	3	4	7	8	9	10	11	12	14	15	18	21
tetO at O_L1	A	A	A	A	A	A	A	A	A	A	A	B
SD for *tetR*	A	A	A	A	A	A	A	A	A	A	D	A
lacO at P_{RM}	C	C	C	C	C	C	C	C	C	C	C	C
P_{RM}*	U	U	W	W	W	W	W	W	W	W	W	W
tetO at O_R1	B	B	A	B	B	C	C	C	C	C	C	A
lacO at P_R	B	C	C	C	C	A	A	B	C	C	A	C
SD for *lacI*	F	C	F	B	F	C	F	F	B	C	C	C
Plaque formation − IPTG†	+	+	+	+	+	−	+	+	+	+	+	+
Plaque formation + 10^{-5} M IPTG†	+	+	+	+	+	+	+	+	+	+	+	+
Lysogenization‡	−	+	−	+	+	+	+	+	+	+	−	+
Single lysogens§	N	+	N	+	−	+	+	+	−	−	N	+

N, not applicable. All isolates carried allele A of the *lacO* at P_L.

*W, variant of λ P_{RM} with the −35 and −10 regions of λ P_{RM} and a *lacO* allele between these regions; U, the same promoter with the *prmup-1* mutation in the −35 region.

†+, plaque formation; −, no plaque formation.

‡+, lysogen formation −, no lysogen formation.

§+, single lysogen formation; −, no single lysogen formation.

contain the strongest SD for *tetR* and the weakest *lacO* at O_R3. All except TL21 also contain the strongest *tetO* at O_L. Formation of stable lysogens by TL phages suggests that TetR can substitute for CI. However, the situation is more complex, as we next describe.

Genetic Switch. We tested whether lysogens of TL phages could carry out prophage induction. In λ, this process occurs upon induction of the host SOS system, which leads to RecA-mediated cleavage of CI. Tet repressor function is unaffected by RecA, but can be modulated by addition of aTc to the growth medium. Accordingly, varying amounts of aTc were added, and phage production was measured as a function of time. All lysogens gave induction, indicating that they could switch from the lysogenic to the lytic state like the WT λ (Fig. 2*A*). Phage production began between 30 and 40 min after adding aTc (Fig. 2*B*); because λ begins producing phage at ≈35 min after infection under our conditions (1), this result suggests that switching occurred almost immediately upon aTc addition. For many variants, 1×10^{-7} M aTc was enough for full induction (Fig. 2*B*).

Unexpectedly, all lysogens also induced efficiently upon addition of IPTG, which has no effect on TetR but weakens the binding of LacR (Fig. 2*A*). Phage production after IPTG addition had the same kinetics (data not shown) as seen (Fig. 2*B*) after aTc induction. These findings strongly suggest that LacR is present during the lysogenic state; that it is continuously required to maintain the lysogenic state; and that the repression by TetR is too weak to maintain the lysogenic state by itself. In λ, Cro cannot repress P_R simultaneously with CI, because both proteins bind to the same operators. However, because TetR and LacR bind to different and spatially separated operators in the TL phage, both should be able to bind simultaneously. Our findings suggest that they do so, and that binding of both repressors is required for maintenance of the lysogenic state.

Changes in cis-acting sites can be expected to alter the operation of a regulatory circuit. We identified several pairs of variants in which a single site change of *tetO* or SD caused an altered phenotype for lysogenization (SI Fig. 5). In most cases, the change should affect repression of P_R. Two pairs affect repression by TetR, in each case differing only in the *tetO* sites in O_R1. TL8 has a stronger operator than TL14, which should cause stronger repression of P_R; TL8 could form a single lysogen, but TL14 could not. In contrast, TL9 has a weaker operator than TL7; TL9 could establish the lysogenic state, but TL7 could not. Taken together, these findings suggest that there is an optimal level of repression of P_R by TetR. We surmise that this level of repression is optimized for expression of LacR. In another pair, TL10 has a strong LacR binding site at P_R, whereas that in TL15 is weak; TL10, but not TL15, could form stable single lysogens. Finally, two pairs (TL8 vs. TL9, TL5 vs. TL6) differed only in the SD sequence for the *lacI* gene. In both pairs, the SD sequence of the first phage is stronger, presumably leading to higher levels of LacR, and the first phage could form a single lysogen, but the second could not. Again, this finding strongly suggests that LacR plays an important role in maintaining a stable lysogenic state; presumably less P_R expression is required to make functional LacR levels in TL8 or TL5 than in TL9 or TL6, because the mRNA is translated more efficiently. Taken together, these results suggest that strong repression of P_R is important for maintenance of the lysogenic state, and that both repressors are required for stable lysogeny to occur.

Influence of the Distance Between P_R and P_{RM}. We asked whether the requirement for both repressors reflected the arrangement of cis-acting sites in our constructs. One or both repressors might have actions other than the intended repressive effects on the adjacent promoters. To test the possibility, we altered the relationships among the cis-acting sites by making TL12 variants with 5-, 10-, or 15-bp insertions between the −35 regions of P_R and P_{RM}, that is, in the middle of the relict O_R2 site. Like the parental TL12 phage, all three variants were able to form stable single lysogens. These results argue against nonlocal effects of TetR and LacR on their respective promoters. In addition, they indicate that TetR almost certainly does not support positive autoregulation of P_{RM}.

Induction by Transient Addition of IPTG and aTc. Prophage induction needs derepression of P_R and P_L to transcribe the lytic genes. How long must derepression occur for switching to the lytic state? In λ, this issue was addressed (21) by exposing a λ*c*I857*ts* lysogen to high temperatures for various lengths of time, affording transient inactivation of CI (the mutant CI protein regains function when returned to low temperature). Inactivation for ≤5 min does not allow switching, but times ≥7.5 min lead to efficient switching. To test how long it takes for switching of TL phages to become irreversible, we exposed lysogens of TL8 and TL21 to 10^{-8} M aTc or 10^{-3} M IPTG; aliquots were then shifted to 10^{-11} M aTc or 10^{-6} M IPTG, respectively, at intervals over the next two hours. Shifting at ≥10 min resulted in good burst sizes (data

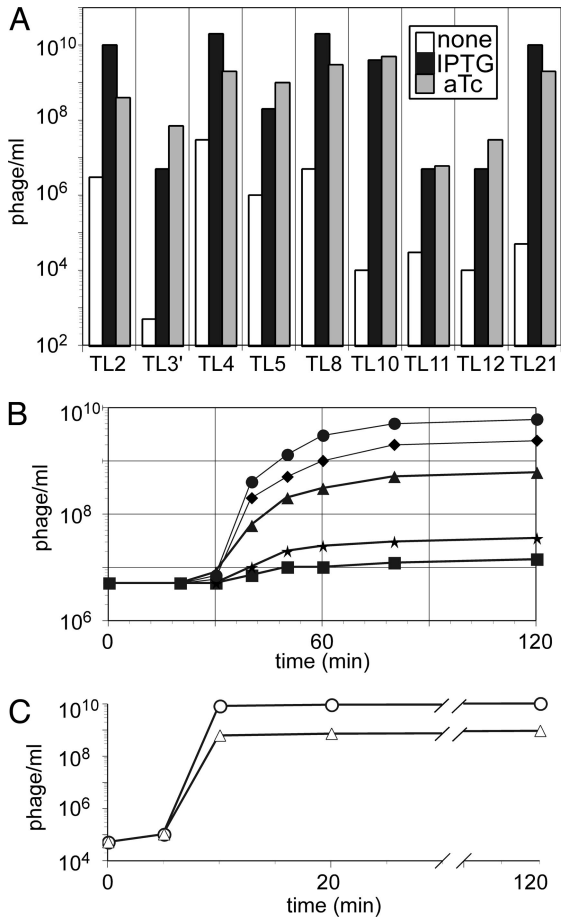

Fig. 2. Prophage induction by aTc and IPTG. Single lysogens of each variant in strain JL6142 were grown exponentially to 10^8 cells per ml and treated as described for each panel. (*A*) Induction by aTc and IPTG. Cultures were exposed to10^{-3} M IPTG (black bar), 10^{-7} M aTc (gray bar), or no agent (white bar), shaken 2 h, treated with $CHCl_3$, and titered. (*B*) Time course of aTc induction. A lysogen of TL8 was exposed to aTc at 0 (squares), 10^{-10} M (stars), 10^{-9} M (triangles), 10^{-8} M (diamonds), or 10^{-7} M (circles). Aliquots were treated with $CHCl_3$ at indicated times and titered. In other experiments (data not shown), 10^{-6} M aTc led to the same yield of phage as 10^{-7} M aTc. (*C*) Prophage induction after transient exposure to aTc (triangles) and IPTG (circles). A lysogen of TL21 was exposed to 10^{-8} M aTc (triangles) or 10^{-3} M IPTG (circles) at time 0. At the indicated times, aliquots were diluted 1,000-fold, and growth was continued; after 120 min, all samples were treated with $CHCl_3$ and titered. A TL8 lysogen gave the same kinetics as shown here for TL21 (data not shown), except that the titer at time 0 was higher (also see *A*).

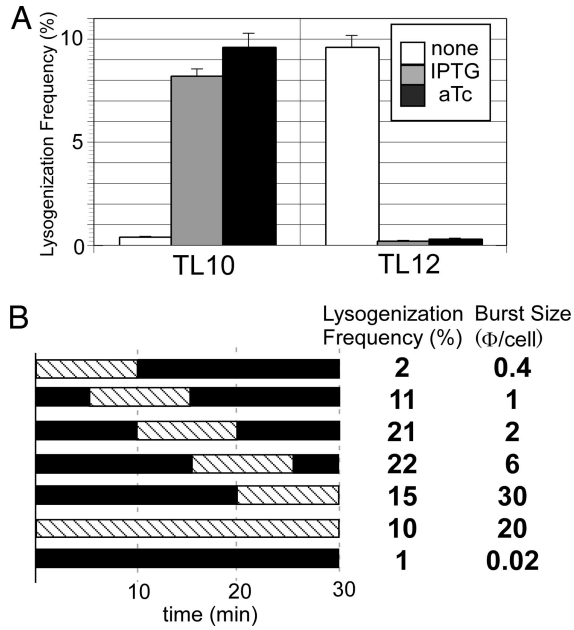

Fig. 3. Effect of IPTG on behavior of TL phage. (*A*) Lysogenization frequency in JL7902 without (white bar) or with 10^{-4} M IPTG (gray bar) and 10^{-8} M aTc (black bar). (*B*) Lysogenization frequency (in JL7902) and burst size (in JL6142) of TL10 in an IPTG concentration-shift experiment. Black bar, 10^{-7} M IPTG; striped bar, 10^{-5} M IPTG.

for TL21 are in Fig. 2*C*). We conclude that derepression of P_R and/or P_L for 10 min sufficed to make the switch irreversible.

Stability of the Lysogenic State. A simple assay for the intrinsic stability of the lysogenic state is to measure the level of free phage in cultures of lysogenic cells. Spontaneous switching leads to lytic growth and production of free phage. In *recA*$^+$ lysogens of λ, spontaneous SOS induction leads to high free phage levels, obscuring the intrinsic stability of the lysogenic state. In *recA*$^-$ lysogens, we find WT free phage at ≈2–5 phage per ml (J.W.L. and C. B. Michalowski, unpublished data), indicating that switching from the lysogenic state to the lytic state occurs, at most, at a rate of 2–10 × 10^{-9} per cell per generation, a value below the spontaneous mutation rate.

We measured the stability of lysogens of most of the TL phages that formed single lysogens. Because induction of TL phages is not RecA-dependent, the level of free phage in cultures of lysogens directly reflects the stability of the circuits. Lysogens produced free phages at widely varying titers (Fig. 2*A*). TL4 and TL8, with the weakest *lacO* site at P_R, were the least stable. TL21, with the strongest *tetO* at O_R1, and TL10 and TL11, with the strongest *lacO* at P_R, were more stable ($<10^5$ phage per ml); however, the low value for TL10 might reflect its poor lytic growth in the absence of aTc or IPTG (see *The Lysis-Lysogeny Decision*), rather than its intrinsic switching rate. These results indicate once again that the repression strength of P_R is important for the stability of this state. At the same time, because some P_R expression is needed to make LacR, this repression cannot be complete. We surmise that an optimum level of P_R expression is necessary to stabilize the lysogenic state.

We found a very stable lysogen. TL3 was unable to lysogenize. A derivative of TL3, termed TL3′, was isolated that could do so. TL3′ contains a change in P_L (−10 region GATACT to GATTCT) that is probably a down-mutation, because it changes a base away from consensus. TL3′ lysogens produced free phages at a very low rate (≈5 × 10^2/ml). In λ, most free phage arising from *recA*$^-$ lysogens carry a destabilizing mutation (J.W.L. and C. B. Michalowski, unpublished data); in contrast, most of the free phage arising from a TL3′ lysogen do not contain mutations that destabilize TL3′ (data not shown), indicating that their titer accurately reflects the intrinsic stability of TL3′. We speculate that the P_L mutation decreases the amount of *N* protein made from P_L, and that reduced *N* levels somehow stabilize the phage, perhaps by reducing the amount of read-through at the T_R1 terminator.

The Lysis-Lysogeny Decision. As described in the Introduction, proper operation of the λ circuitry requires a balance between the two states of the system. We found that the balance of the TL phage system could be perturbed by adding aTc or IPTG. We measured lysogenization frequency after single infection, using an *hfl*$^-$ host (see next paragraph) to increase this frequency, in the absence or presence of aTc or IPTG. For most variants, aTc and IPTG decreased the efficiency. For example, ≈9% of TL12

chose the lysogenic pathway without inducers (Fig. 3*A Right*). Either aTc or IPTG decreased the lysogenization frequency to <1%. Presumably these agents increased expression of the lytic promoters, favoring lytic growth.

In λ, CII protein favors lysogeny by activating the P_{RE}, P_{aQ} and P_I promoters (22). CII is unstable in WT host strains, being degraded by the FtsH protease (23), and is stabilized in an *hfl*$^-$ host. We found that lysogenization frequencies after single infection in an *hfl*$^+$ host were markedly lower, at least for TL21 and TL8, than in an *hfl*$^-$ host; respective values for λ, TL21, and TL8 were 1.2%, 0.02%, and 0.003% for *hfl*$^+$, and 60%, 9%, and 6% for *hfl*$^-$. We conclude that CII stimulates the lysogenic pathway in TL phages, as it does in λ.

For one variant, TL10, we found by contrast that inducers increased greatly the lysogenization frequency in the *hfl*$^-$ host (Fig. 3*A Left*). One possible explanation for this finding comes from a comparison with TL15, which differs from TL10 only in the *lacO* sites in P_R. TL10 and TL15 have the strongest and weakest operator, respectively. TL10 was unique among our isolates in not being able to form plaques in the absence of both IPTG and aTc. These results suggest that P_R is repressed too much in TL10 early after infection, blocking both the lytic and lysogenic pathways.

Temporal Analysis of Repressor Activity. To gain some insight into the nature of the block to TL10 growth and lysogenization, and whether it was the same for both pathways, we asked when the block occurred using an IPTG pulse experiment. We applied ten-minute pulses of IPTG at various times after infection, and measured lysogenization frequencies and ability to grow lytically. (Fig. 3*B*). Cells were initially grown at 10^{-7} M IPTG after phage infection. IPTG concentration was shifted to 10^{-5} M IPTG for 10 min at various times during 30 min. Interestingly, there was a difference in timing between permissive conditions for the lysogenic and lytic pathways, suggesting that the blocks in the two pathways operated at somewhat different times. In tests of lysogenization frequency, carried out in an *hfl*$^-$ host (Fig. 3*B*), pulses before 10 min had no effect on the ability to lysogenize, whereas pulses at later times, even after 20 min, markedly increased the lysogenization efficiency. In measurements of lytic growth, carried out in an *hfl*$^+$ host to favor the lytic response (Fig. 3*B*), we found that pulses before 20 min had no effect on the burst size, whereas those after 20 min increased the burst size. Possibly the difference in timing reflects the observation with λ (22) that Q function, which is required for lytic growth, appears substantially later than that of CII, which favors the lysogenic pathway.

Evolution of TL Phages. One advantage of the λ system is that we can easily isolate variants with altered behavior using genetic selections. Above we described a variant of TL3 that could lysogenize. Here we tested whether TL10, which could not make plaques in the absence of inducers, could evolve under selective pressure. We isolated 10 independent variants that could form plaques. Of these variants, four had changes in the *lacO* in P_R. Two had changes in *lacI* (P127→Q and Q291→K) that confer partial loss of repressor function (24). Two had changes in TetR [nonsense codon at residue 43 and T40A, changing a residue directly interacting with DNA (17)]; at least the nonsense mutation should abolish TetR function. In the remaining two mutants, which formed tiny plaques, no mutations were identified in *lacI*, *tetR*, or the O_R and O_L regions. These results imply once again that the growth defect of TL10 resulted from overly strong repression of P_R by both TetR and LacR, leading to restriction of early lytic gene expression., In support of this conclusion, TL18, which differs from TL10 only by having a weaker SD sequence for TetR expression and presumably had less TetR and therefore weaker TetR function, could form plaques in the absence of IPTG and aTc.

Discussion

Altered Wiring Diagram for TL Phages. Lysogens of TL phages were induced by aTc or by IPTG (Fig. 2). These findings indicate that both repressors must function to maintain stable lysogeny. LacR does not simply serve a backup role, guarding against occasional leaky expression of a lytic promoter; addition of IPTG for ten minutes led to efficient switching, implying that LacR was required continuously to maintain repression. In λ, by contrast, Cro does not play a role in maintenance of the lysogenic state. Accordingly, the TL phages display an altered wiring diagram from that of λ.

We believe it unlikely that the inability of TetR to work by itself results from its inability to afford complete repression. In Tn*10*, the O1 operator has the same relationship to the −35 and −10 regions of the P_A promoter as used here with P_R and P_L, and TetR can repress the P_A promoter 500-fold (17).

We suggest instead that the altered circuitry is a necessary consequence of losing some or all of the features of CI protein that afford nonlinearity and positive feedback to its action. These features make the DNA-binding curve for CI markedly nonlinear, allowing transcription of the lytic promoters at low levels of CI and conferring switch-like repression at higher CI (25). It is unknown whether dimerization of TetR is weak enough to afford some nonlinearity. It is also formally possible that some unknown feature of TetR, or a physical interaction between bound TetR and LacR, can compensate for some or all of the missing features of CI, providing the nonlinearity and/or positive feedback conferred by CI, at least in the context of the TL phage circuitry, but we believe this possibility to be unlikely. With these caveats, it is likely that the TetR DNA-binding curve is more linear than that of CI.

A likely consequence of increased linearity in the binding curve is that the ability to achieve strong repression of one or both lytic promoters at high TetR levels may be incompatible with expression during lytic growth at low TetR levels; accordingly, by selecting mostly for phage that could grow lytically we might have eliminated phage with tight repression of P_L or P_R. One line of evidence consistent with this idea is that, of all of the phages able to form stable single lysogens, only TL21 had the strongest *tetO* site at P_R. We surmise that loss of tight repression can account for the reduced stability of TL phage lysogens relative to that of λ.

It would be of interest to know whether this artificial circuit can exhibit bistable behavior. As noted in the Introduction, when the lytic pathway is blocked by mutation the λ circuitry can persist in an anti-immune state, in which Cro has gained the upper hand and continues to repress CI. Bistability may not be possible with this circuit, because both repressors are required to maintain the lysogenic state (6).

Evolution and System Parameters. Our findings are compatible with the model (1, 26) that gene regulatory circuits have gained refinements, such as those conferring nonlinearity or positive feedback, during the course of evolution. In this view, such features offer selective advantages by providing useful systems properties such as redundancy, robustness, and stability. In λ, we speculate that the nonlinearity and positive feedback provided by CI contribute to the great stability of λ lysogens.

Our findings also provide further support for our proposal (3) that another type of refinement during evolution is changes in system parameters, such as promoter strengths or affinities of DNA-binding proteins for their binding sites. We found many pairs of TL phages in which changes in a single cis-acting site led to altered behavior (see *Results* and SI Fig. 5). Clearly, changes in the affinities of TetR or LacR for their sites, or changes in

their translation efficiencies, had marked effects on systems properties.

Selection for altered behavior also led to changes in system parameters. A down-promoter mutation of P_L in TL3 allowed the TL3′ variant to form stable lysogens. Among variants of TL10 that could form plaques in the absence of inducers, half were in a particular cis-acting site, even though the target size for mutagenesis of the proteins is far larger. These findings provide a clear-cut example of the model (10) that regulatory circuits evolve, at least in large part, by alterations in the cis-acting sites, because mutation of the proteins is far more likely to have pleiotropic effects on multiple target genes. Although we did find mutations in *lacI* and *tetR*, these changes weakened or abolished repressor function rather than affording differential regulation of particular promoters; at the circuit level they are likely functionally equivalent to providing less repressor by changes in cis-acting sites.

Modularity in Gene Regulatory Circuits. For the sake of discussion, we define a regulatory module as a regulatory protein, its cis-acting sites, and the consequences of binding (2). We suggested previously (2) that complex circuits can arise by random associations of regulatory modules, and that λ retains such an organization, because we were able to replace Cro with LacR. Our current evidence provides further support for this view.

In λ, CI and Cro modules are perhaps unusual among regulatory circuits, in that they share the same set of cis-acting cites. It is plausible that, even if these modules were clearly distinct when the system arose, sharing the same sites might have led to breakdown of the separate identities of the two modules. Our evidence that the CI module is also replaceable suggests that, to the contrary, the λ circuit has largely retained its modularity.

New Toolkits for Bacteriophage Engineering. Our success at isolating phages whose behavior is regulated by small molecule inducers suggests that this approach could be useful in the field of biological engineering and medicine. Phages are usable for antibacterial therapy (27); they are highly specific to bacterial species; they are nontoxic to animals and plants; and they usually kill their target microbes. Phages could be developed whose life cycle is regulated either by small molecules provided as a therapeutic treatment or present in particular environments, or by protein cofactors of specific strains. Such phages might be used to detect environmental conditions or treat infections with pathogens.

Materials and Methods

Media and Chemicals. Tryptone broth, LB, LBGM, LBMM, and TMG were as described (1) and were supplemented with antibiotics as appropriate; kanamycin was at 10 μg/ml. Oligonucleotides were from Qiagen (Alameda, CA) and are listed in SI Table 4.

Phage and Bacterial Strains and Plasmids. Phage strain λJL351 was used as WT (2, 26) and was modified for use as a cloning vector as described in *SI Materials and Methods* and SI Fig. 6. Bacterial strain JL6142 Δ(*lacIPOZYA*) (2), a derivative of JL2497, was used as WT. Strain JL7223 was an *hfl*$^-$ derivative of JL6142 (2); it contains Tn*10*, which includes the *tetR* gene. Strain JL7902, a Tet^S derivative of JL7223, was isolated as described (28).

Screening of TL Phages. Phages of this library (made as described in *SI Materials and Methods* and SI Fig. 7) were plated on JL6142 on tryptone plates without or with IPTG at 10^{-5} M or 10^{-4} M. Turbid plaques were purified and analyzed by PCR; isolates with a fragment of the expected size were further characterized. In a separate selection, JL7223 was infected at a multiplicity of infection of ≈0.5 with phages from the pool, and Kan^R colonies were isolated. Segments of TL phages were amplified by PCR of plaques with primers A17 and A18 (O_L), A95 and A96 (*tetR*), A152 and A153 (O_R), or A20 and A80 (*lacI*). PCR products were sequenced by automated cycle sequencing at the Division of Biotechnology, University of Arizona. Isolation of variants with insertions in O_R2 is described in SI Fig. 8.

Test for Single Lysogens. This test was carried out as described (29). In this test, a single lysogen gives a single PCR product; presence of a second product indicates either the presence of multiple prophages or the presence of replicating λ DNA resulting from spontaneous switching of a relatively unstable lysogen.

Lysogenization Tests. To test for lysogenization frequency after single infection, JL6142 or JL7902, as indicated, was grown in LBMM to 2×10^8/ml, centrifuged, concentrated 10-fold in TMG, and mixed with phage at a multiplicity of infection of ≈0.01; after 20 min at room temperature, aliquots were diluted into LBGM containing 0, 10^{-5}, 10^{-4}, or 10^{-3} M IPTG, shaken 30 min at 37°C, and plated in 3 ml of top agar plus 300 μl of LB on tryptone plates containing 10 μg/ml kanamycin. For the IPTG concentration-shift assay (Fig. 3*B*), after LBGM was added as described above, IPTG was added at the indicated times, and the solution was diluted 100-fold with LBGM after a further 10 min.

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