Cooperative DNA binding by CI repressor is dispensable in a phage λ variant

Andrea C. Babić and John W. Little*

Department of Biochemistry and Molecular Biophysics and Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721

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Complex gene regulatory circuits contain many interacting components. In principle, all of these components and interactions may be essential to the function of the circuit. Alternatively, some of them may be refinements to a simpler version of the circuit that improve its fitness. In this work, we have tested whether a particular property of a critical regulatory protein, CI, is essential to the behavior of the phage λ regulatory circuit. In the lysogenic state, CI represses the expression of the lytic genes, allowing a stable lysogenic state, by binding cooperatively to six operators. A mutant phage lacking cooperativity because of a change in cI could not form stable lysogens; however, this defect could be suppressed by the addition of mutations that altered two *cis*-acting sites but did not restore cooperativity. The resulting triple mutant was able to grow lytically, form stable single lysogens, and switch to lytic growth upon prophage induction, showing a threshold response in switching similar to that of wild-type λ. We conclude that cooperative DNA binding by CI is not essential for these properties of the λ circuitry, provided that suppressors increase the level of CI. Unlike wild-type lysogens, mutant lysogens were somewhat unstable under certain growth conditions. We surmise that cooperativity is a refinement to a more basic circuit, and that it affords increased stability to the lysogenic state in response to environmental variations.

cooperativity | evolution of regulatory circuitry | gene regulatory circuit | systems biology | prophage induction

Complex gene regulatory circuits include many components and interactions. It is likely that these features are advantageous and have been selected for during the course of evolution. However, certain features may be refinements to the system rather than essential to its operation. In this view, a complex system might have arisen in a much simpler form, with refinements being added later as the system evolved toward its present form. This idea predicts that existing circuits might tolerate the removal of certain features (perhaps with the addition of suppressors) and still show qualitatively normal behavior. In this work, we have tested this prediction in a well characterized complex circuit, the bistable switch of phage λ, by removing one feature long believed to be essential for its proper operation.

A cell infected with λ can follow either of two pathways, the lytic or lysogenic pathways. In the lytic pathway, the phage DNA replicates, and ≈100 new virions are made and released upon cell lysis. In the lysogenic pathway, the viral genome integrates into the host chromosome, and the lytic genes are repressed by the action of CI protein. This pathway leads to a stable regulatory state, the lysogenic state. In this state, CI binds tightly to two operators in the O_R region, O_R1 and O_R2, repressing the expression of the lytic P_R promoter and stimulating its own expression from another promoter, P_{RM} (1).

Although the lysogenic state is highly stable and can be maintained indefinitely, lysogenic cells can change to the lytic state in a process called prophage induction, or the genetic switch. This process is triggered when the host SOS regulatory system is activated by DNA damage (2, 3). Damage leads to activation of RecA protein, which interacts with CI and stimulates a latent self-cleavage activity of CI (4). Cleavage of CI inactivates it as a repressor; when levels of intact CI fall to low values, transcription from the lytic promoters P_L and P_R begins, allowing the lytic pathway to proceed. Prophage induction displays threshold behavior: at low doses of an inducing treatment, such as UV irradiation, only a small fraction of the lysogens switch. However, above a critical dose of inducing treatment, a dose termed the set point, the majority of lysogens switch.

CI binds cooperatively to adjacent operator sites. In the O_R region (Fig. 1*B*), O_R1 is a strong binding site. O_R2 is intrinsically a weak binding site, but CI binds ≈40-fold more tightly to O_R2 if CI is also bound to O_R1 (5, 6). Cooperative binding increases the occupancy of both O_R1 and O_R2 at a given concentration of CI, leading to greater repression of P_R and stimulation of its own expression from P_{RM}. In addition, a higher-order form of cooperative binding can occur, in which CI bound cooperatively to O_R1 and O_R2 interacts with CI bound at two distant sites, O_L1 and O_L2, forming a long-range loop (7–9). Finally, further cooperative interactions lead to occupancy of O_L3 and O_R3, causing repression of P_{RM} (Fig. 1*D*).

It has long been believed that cooperative binding is crucial to the operation of the λ regulatory circuit, particularly for prophage induction (1, 10, 11). Cooperativity provides a form of nonlinearity, such that occupancy of the operators is substantially more than proportional to the concentration of CI. Nonlinearity is also provided by the fact that CI dimerizes rather weakly (12). Acting together, cooperativity and weak dimerization confer a steep sigmoid shape to the binding curve of CI for its operators. Accordingly, as CI levels drop during prophage induction, occupancy of the operators drops rather abruptly over a relatively narrow range of CI concentrations, perhaps facilitating switching to the lytic state.

To date, *in vivo* analysis of CI cooperativity has been carried out in simplified systems in which CI expression and levels are uncoupled from its regulatory activities (13–16). Although necessary to analyze causal connections of this system, this approach cannot reveal the effects of cooperativity on the intact circuitry, because it removes positive and negative autoregulation of *c*I. In this work, our aim was to test whether CI cooperativity is essential to the circuitry in the intact phage.

We found that a mutant phage deficient in cooperativity was unable to form stable lysogens. We isolated variants that restored the ability to form lysogens, while not altering the cooperativity deficiency of the phage, and tested them for qualitatively normal behavior, which we defined by three criteria. First was lytic growth after infection, not a demanding criterion because CI plays no role in lytic growth. Second was the

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*To whom correspondence should be addressed at: 1007 East Lowell Street, Life Sciences South Building, Tucson, AZ 85721. E-mail: jlittle@u.arizona.edu.

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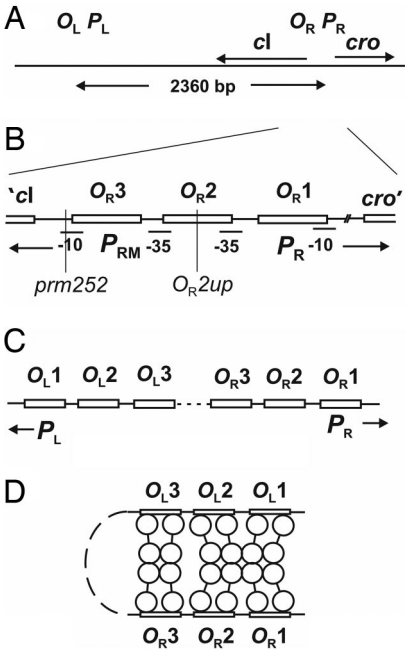

Fig. 1. Maps of λ. (*A*) Diagram of the λ immunity region. Location of O_R and O_L regions, and the *cI* and *cro* genes, are shown. CI and Cro can bind to a total of six operators, three in each operator region (see *C*) (1). Map is not quite to scale. (*B*) Details of O_R region. The promoters controlling *cI* and *cro* expression, P_{RM} and P_R, respectively, are shown. Map is to scale. The *prm252* mutation changes the −10 region of P_{RM} from TAGATT to TAGACT; *O_R2up* changes O_R2 to CAACACGCGCGGTGTTA (underlined base is A in wild type). (*C*) Map of O_R and O_L. Operators in O_L and in O_R, and the two early lytic promoters P_L and P_R, are shown. (*D*) CI-mediated loop between O_L and O_R. The complex illustrated forms in two steps (8). First, at relatively low CI levels, cooperatively bound pairs of dimers at O_L and O_R interact, forming an octamer of CI (data not shown). This higher-order cooperative interaction increases occupancy of these operators, repression of P_L and P_R, and activation of P_{RM} at low CI levels. Second, as CI levels increase further, CI binds cooperatively to O_L3 and O_R3, forming a complex with 12 CI molecules. Occupancy of O_R3 blocks expression from P_{RM} (8). Details of the protein–protein interactions in this complex are not known.

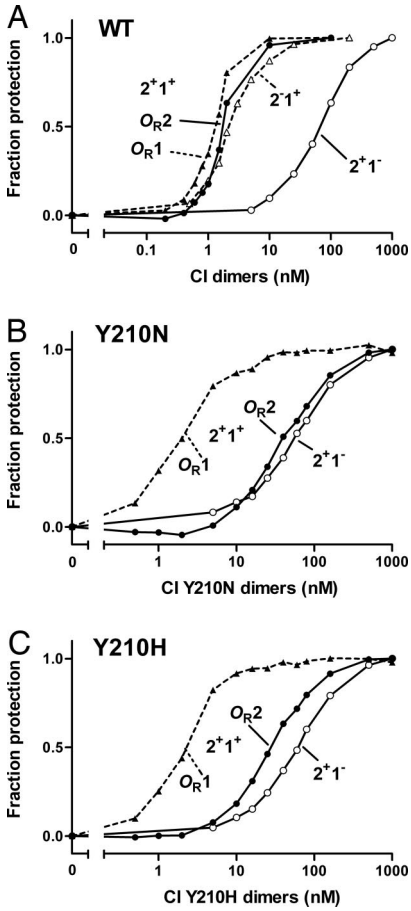

Fig. 2. Cooperativity defect of Y210N and Y210H mutant proteins. Binding curves for wild-type CI (*A*), CI Y210N (*B*), and CI Y210H (*C*). Binding to O_R2 and O_R1 is shown by solid and dashed lines, respectively; binding to a template with only one binding site or with both binding sites is shown by open and closed symbols, respectively. Cooperativity causes the curve for O_R2 to shift to the left on the template with both sites (compare open and closed symbols for solid lines); a small shift (as in *B* and *C*) reflects a cooperativity defect. The $2^+1^=$ and $2^=1^+$ templates are denoted by 2^+1^- and 2^-1^+, respectively; the latter template was used only for WT CI (see *Materials and Methods*). Percent occupancy was determined by quantitative DNase I footprinting by using amounts of CI or mutant protein giving the indicated concentrations of dimers (see *Materials and Methods*). Affinity is given by the concentration of dimers affording 50% occupancy.

ability to form stable single lysogens. Third was the ability to switch from lysogenic to lytic growth and give a relatively large burst size. We describe variants of λ that lack CI cooperativity and exhibit qualitatively normal behavior by these criteria.

Results

Choice of Cooperativity-Deficient Alleles. Although several cooperativity mutants have been isolated (14–16), most of the mutant alleles would be expected, or have been shown biochemically (17), to affect other aspects of CI function, including dimerization and specific cleavage. We used substitutions of Tyr-210 for several reasons. This residue is not in the dimerization interface (18) or likely to affect CI cleavage (4, 19). Several cooperativity-deficient mutants had been isolated by genetic screens, including Y210N (14), Y210H (16), and Y210C (15). Not all changes in Tyr-210 lead to a defect in cooperativity; our evidence suggests that the *c*I Y210F mutation did not markedly reduce cooperativity [see the supporting information (SI)].

The Y210H mutant protein has been biochemically shown to be deficient in cooperativity (17). We designed a simplified DNase I footprinting assay to detect small degrees of cooperativity (see *Materials and Methods*). With this assay, we found that purified Y210N and Y210H mutant proteins were almost completely deficient in cooperative binding (Fig. 2 *B* and *C*), showing cooperativity parameters of ≈1.4 and ≈2, respectively, compared with a value of ≈70 for wild type (Fig. 2*A*). We also found that both mutant proteins showed approximately the same rates of RecA-mediated cleavage as that of wild-type CI (data not shown), verifying our expectation that these alleles would not affect cleavage.

We crossed both alleles onto a derivative of wild-type λ bearing a kanamycin marker. The λ *c*I Y210H phage formed faintly turbid plaques and unstable polylysogens (lysogens with several tandem copies of the prophage). In contrast, the *c*I Y210N phage, λJL506, formed clear plaques, and no lysogens could be isolated by streaking onto kanamycin plates from the center of the plaque. We conclude that cooperativity is necessary to establish and/or maintain the lysogenic state in a phage that is otherwise wild type.

Lysogenizing Variants of λ *c*I Y210N. Because λ JL506 *c*I Y210N could not lysogenize, we isolated spontaneously arising variants that could do so by selecting for kanamycin-resistant cells in a population of infected cells. This selection was followed by

enrichment for phage that could undergo prophage induction (see *Materials and Methods*). This procedure identified mutations lying in *c*I, *cro*, O_R3, and P_{RM} (detailed in the SI). None of the suppressor mutations should be able to restore cooperativity. We conclude that CI cooperativity is dispensable for establishment and maintenance of the lysogenic state.

However, each of these isolates had defects in lytic growth or in prophage induction. Our inability to isolate spontaneous variants of λJL506 fulfilling our criteria for normal behavior suggested that such mutants might not be able to arise by a single mutational change. Therefore, we designed a combination of suppressors that might restore qualitatively wild-type behavior to a phage deficient in cooperativity.

Design of a Cooperativity-Deficient Phage with Qualitatively Wild-Type Behavior. Among the suppressors were two mutations that increased the strength of P_{RM}. λJL508 *c*I Y210N *prmup-1* has a strong up-promoter mutation in P_{RM} that increases its basal activity 10-fold and its stimulated activity 4- to 5-fold (20). This phage was able to form stable single lysogens, but these had a severe defect in lytic growth after prophage induction, giving a burst of $\ll$0.1 phage per cell. λJL510 *c*I Y210N *prm252* has a weaker P_{RM} up-mutation that increases the basal and activated strength of P_{RM} ≈6-fold and ≈2.5-fold, respectively (C. B. Michalowski and J.W.L., unpublished data). This phage was unable to form single lysogens, and polylysogens gave a low burst of phage after UV induction (≈5 phage per cell). These findings suggested that, at the higher CI gene dosage in a polylysogen, enough CI was made to cause occupancy of O_R2 and to result in higher CI levels due to positive feedback. Therefore, we reasoned that, if CI could bind more tightly to O_R2, then P_{RM} or a P_{RM} variant might be expressed at stimulated levels. CI levels would then be elevated sufficiently in the presence of a relatively weak up-promoter mutation, such as *prm252*, to allow for stable single lysogens. Accordingly, a variant of λ JL510 Y210N *prm252* was created with an additional O_R2 mutation.

A mutation that we term O_R2up (Fig. 1*B*) was obtained in a previous selection (J.W.L. and K. Newell, unpublished data). Its properties in that selection suggested that it altered the balance between CI and Cro in favor of CI. Because a comparable change in O_R1 increases the affinity of CI for that site (21), we measured the affinity of CI Y210N protein for the O_R2up site by using footprinting and found that the intrinsic affinity for this mutant site was increased ≈5-fold (data not shown). Accordingly, we made a triple-mutant phage, λJL516 *c*I Y210N *prm252* O_R2up.

Cooperativity Mutant with Nearly Normal Behavior. This triple-mutant phage formed plaques that were the same size but somewhat less turbid than those of the wild type. Its burst size was approximately the same as that of the wild type (60 and 59 for wild type and mutant, respectively). It also formed stable single lysogens. Importantly, these lysogens behaved nearly identically to wild-type lysogens in prophage induction (Fig. 3*A*), showing threshold behavior, a set point (UV dose giving half-maximal phage production) the same as that of wild type, and a phage yield slightly lower than that of wild type. We conclude that cooperativity is not a necessary feature of the λ regulatory circuitry, provided that a cooperativity defect is suppressed by mutations increasing the level of CI. We also conclude that CI cooperativity is not primarily responsible for the threshold behavior of the switch.

In the lysis-lysogeny decision, λJL516 showed a reduced frequency of lysogenization, 19% versus 40% for wild type. Because λJL516 forms stable single lysogens, it is unlikely that this reduced value results from a defect in maintaining the lysogenic state. Instead, we suggest that it results from impaired CI function during the establishment phase (see *Discussion*).

As outlined above, we expected that the level of CI in λJL516 lysogens would be higher than that shown by wild type to give efficient repression of P_L and P_R, as well as occupancy of O_R2 (and stimulation of P_{RM}) in the absence of cooperativity. We used an ELISA to test this expectation and found that lysogens had approximately 4 times the level of CI as that found in a wild-type lysogen (data not shown). Therefore, increasing the amount of CI protein, together with an increased affinity of CI for O_R2, largely compensated for the loss of cooperativity.

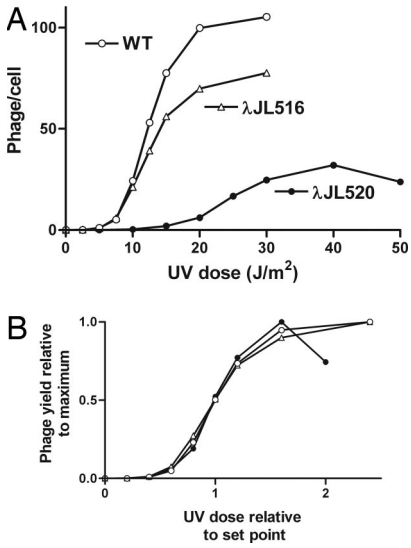

Fig. 3. UV dose–response for prophage induction. (*A*) Exponentially growing cells were irradiated with a range of UV doses, and the titer of released phage was determined, as described in *Materials and Methods*. λJL516 is λ cI Y210N *prm252* O_R2up; λJL520 is λ cI Y210H *prm252* O_R2up. Data are from a single representative experiment. (*B*) Normalized curves. To compare the shape of these curves, data from *A* were replotted by normalizing phage yield relative to the maximum yield for each phage and by normalizing UV dose relative to the set point (dose giving 50% maximum yield). The resulting curves have essentially the same shapes. Symbols are as in *A*.

Finally, we isolated a phage carrying *c*I Y210H and the same two suppressors as used above. This phage, λJL520 *c*I Y210H *prm252* O_R2up, formed turbid plaques of normal size and made stable single lysogens. Lysogens were able to undergo prophage induction with threshold behavior and had a set point higher than that of wild type (Fig. 3*A*). In contrast to λJL516, the burst size of λJL520 was substantially reduced from that of wild type, but the shape of the induction curve was the same (Fig. 3*B*). We have observed (22, 23) that many mutants with a higher set point also show a reduced burst size. We conclude that, like their Y210N counterparts, phages bearing the *c*I Y210H mutation have behavior similar to wild type by these criteria.

Discussion

Our evidence shows that cooperative DNA binding by CI is not required for qualitatively normal behavior of the λ regulatory circuitry. We first address how the suppressors in λJL516 act to suppress the cooperativity defect. We then discuss which aspects of λ physiology may contribute to the threshold response to UV induction. Finally, we consider why cooperativity may have evolved in lambdoid phages.

Mechanisms of Suppression. Mutations in two different *cis*-acting sites, P_{RM} and O_R2, contributed to the relatively normal behavior of λJL516. We believe that these two mutations worked together to increase the CI concentration to levels high enough to compensate for the impaired binding of CI to the O_L and O_R operators. An up-promoter in P_{RM} increased its strength, and a mutation (O_R2up) in O_R2 allowed tighter binding of CI, pre-

sumably leading to a high enough level of occupancy to allow stimulation of P_{RM}, leading to still higher CI levels. In this view, in the absence of the O_R2up mutation, the level of CI was below a threshold value necessary to lead to positive feedback. This interpretation is supported by the finding that λ *c*I Y210N *prm252* could form stable polylysogens, but not single lysogens. The increased gene dosage in a polylysogen would also lead to levels of CI above this threshold. Similarly, λ *c*I Y210N O_R2up could not form single lysogens (data not shown), indicating that the O_R2up mutation on its own could not compensate fully. At the same time, increasing the strength of P_{RM} did not suffice to restore nearly normal behavior. We found that λJL508, a phage with a stronger up-promoter, *prmup-1*, could form stable single lysogens, but that these gave almost no phage upon UV induction. Preliminary evidence (data not shown) suggests that this may result from an aberrant switching process after UV induction; although some late gene expression occurs, as evidenced by cell lysis after chloroform treatment, we observed low levels of transcription from P_R and P_L, suggesting that CI continues to be made, at least sporadically, from the unstimulated *prmup-1* promoter.

We found that λJL516 showed a reduced frequency of lysogenization, although it was able to follow the lysogenic pathway with reasonable efficiency. We rationalize this mild defect as follows. In a cell following the lysogenic pathway, CI is initially expressed from the strong promoter P_{RE}, rather than P_{RM} (24). P_{RE} is regulated by the phage CII protein (24, 25). We expect the level of CI provided by P_{RE} to be approximately the same in wild type and λJL516. However, the mutant CI Y210N protein cannot act as effectively as wild-type CI to repress the lytic promoters, making it somewhat more difficult to repress P_R and P_L and to establish the lysogenic state. Possibly, this defect could be suppressed by a mutation that increases the strength of P_{RE} or alters its regulation (for instance, by making CII more stable).

A λJL516 lysogen showed another defect. When the wild-type lysogen and its nonlysogenic parent were grown in tryptone medium, like the LB used here, but lacking yeast extract, their growth rate abruptly slowed at $\approx 10^8$ cells per ml, presumably because of a nutritional downshift. At this stage, growth of single lysogens of λJL516 slowed to a much greater extent, and the culture accumulated polylysogens, suggesting that the lysogenic state was destabilized by this metabolic condition, and that increased gene dosage conferred by multiple prophages could stabilize it. This behavior was not seen with lysogens of λJL520. Its physiological basis is not understood and requires further study. Although it does not affect the conclusions of this work, this finding suggests that a defect in cooperativity makes the phage less fit in other environments, providing selective pressure for altered circuitry (see *Roles of Cooperativity and Evolution of Gene Regulatory Circuitry*).

Threshold Behavior of Prophage Induction and the Role of Cooperativity. The formal similarity between the sigmoid binding curve for CI binding to O_R and the sigmoid shape of the dose–response for prophage induction suggested that cooperativity might contribute to the latter behavior. Our data are inconsistent with a decisive role for cooperativity in threshold behavior, because the induction curves of λJL516 and wild type are almost identical. We suggest several remaining features of the viral and host circuitry that might contribute to threshold behavior.

First, the λ circuitry retains several sources of nonlinearity. These sources include weak dimerization by CI and Cro proteins, positive autoregulation of *c*I, and double-negative feedback between CI and Cro. These forces can help contribute to bistability and a nonlinear response to an input (26). Second, evidence with cleavage of LexA (27) indicates that the initial level of activated RecA is approximately the same at all UV doses over the range used here, but that RecA remains activated for longer times at higher UV doses. It is known (28) that it takes 20–30 min for cells to switch and a comparable time to reduce CI levels to low values. Hence, the timing of RecA activation may also contribute to this nonlinear response.

We found that the set point for the mutant phages with *c*I Y210N and *c*I Y210H differed (Fig. 3). This difference probably did not arise from differences in RecA-mediated cleavage, because both proteins showed wild-type rates for this reaction (data not shown). It is unlikely to result from differences in dimerization, because Y210 lies far from the dimerization interface, and Y210H protein dimerization is normal (29). The simplest interpretation is that the difference arises from the difference in the amount of cooperativity remaining in the two proteins (Fig. 2), although it is formally possible that differences in the *in vivo* properties of the mutant proteins were not detected by *in vitro* tests. With this caveat, we suggest that the difference in set point arises from the higher degree of residual cooperativity of Y210H protein, which will continue to allow expression of *c*I at somewhat lower levels than in the Y210N phage, thereby counteracting RecA-mediated cleavage more effectively at intermediate UV doses. Our previous evidence also shows a correlation between set point and the strength of P_{RM} (22).

Roles of Cooperativity and Evolution of Gene Regulatory Circuitry. It is likely that CI cooperativity evolved early in the history of lambdoid phages, because all lambdoid phages tested have CI cooperativity, with the possible exception of 933W (30). In addition, mutations in corresponding residues affect the cooperativity of different CIs (31). The behavior of phages lacking cooperativity suggests several possible roles for cooperativity in λ regulatory circuitry. First, cooperativity should allow the levels of CI in a lysogen to be lower than in the absence of cooperativity, as found here. This feature may be advantageous in that it might make it easier to induce at a given dose of DNA damage, because there would be less CI to cleave. Second, less CI would be required to establish the lysogenic state after infection, as judged by the reduced lysogenization frequency of λJL516 relative to wild type. Third, cooperativity contributes to switch-like behavior of the P_{RM} promoter. Above a critical level of CI, it favors positive autoregulation of *c*I at P_{RM} by promoting binding to O_R2. Hence, this feature allows the basal level of CI expressed from P_{RM} to be very low at low CI levels, possibly preventing low CI levels from interfering with lytic growth. Our findings are consistent with this speculation. In summary, λ could likely achieve these goals in different ways, and it is probably not possible for us to determine why cooperativity evolved instead of a different solution. However, cooperativity offers an economical solution to all of these issues.

Our previous work (23, 32) has shown that two other aspects of the gene regulatory circuitry are also not necessary for λ to maintain qualitatively normal circuitry, namely the differential affinities of CI and Cro, or the positive control of CI. However, in each case, the detailed behavior of the mutant phage is altered. Efforts to remove both cooperativity and positive control (A.C.B., J.W.L., and C. B. Michalowski, unpublished data) while retaining normal circuitry have not yielded phages that can form stable lysogens. In any case, we suggest that all of these features are refinements that contribute in various ways to the overall behavior of λ, and that they were added to a simpler, more basic form of the circuitry during the course of evolution.

Materials and Methods

Strains and Plasmids. Bacterial strains used in this work were JL2497 (33), a derivative of strain N99 (34) used here as wild type; JL5932 (32) = JL2497 (λJL163); and JL6039 = JL2497 *hflC150*::Tn*10* (22). Phage strains were λJL163 (32), which is λ^+ with a *bor*::*kan* substitution, and the following derivatives of λJL163, most of them made as described below or isolated as

described in *Results*: λJL506 *c*I Y210N; λJL508 *c*I Y210N *prmup-1*; λJL510 *c*I Y210N *prm252*; λJL514 *c*I Y210H; λJL515 *c*I Y210H *v1 v3*; λJL516 *c*I Y210N *prm252* O_R2up; and λJL520 *c*I Y210H *prm252* O_R2up. Plasmids, and construction of plasmids and additional phage strains, are described in the SI.

Chemicals and Enzymes. Restriction enzymes, BSA, polynucleotide kinase, and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA); DNase I was obtained from Promega (Madison, WI); TaqDNA polymerase was obtained from Roche (Indianapolis, IN); Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA); γ-^{32}P ATP was obtained from MP Biomedicals (Solon, OH); Affigel blue, hydroxyapatite, and the protein assay kit were obtained from Bio-Rad (Hercules, CA); phosphocellulose was obtained from Whatman (Clifton, NJ); oligonucleotides were obtained from Midland Certified Reagent (Midland, TX) and Qiagen (Valencia, CA); BugBuster HT Protein Extraction Reagent was obtained from Novagen (Madison, WI); and the ImmunoPure TMB substrate kit was obtained from Pierce (Rockford, IL). Rabbit anti-CI was as described in ref. 35 and was affinity-purified as described in the SI.

Growth Media. LB and tryptone broth were as described in ref. 22. TMG was 10 mM Tris (pH 8), 10 mM $MgSO_4$, and .001% gelatin. Bacto-tryptone, Bacto-agar, and yeast extract were from Difco (Detroit, MI).

Construction of Phages with Cooperativity Mutants. Each phage was isolated in a phage-by-plasmid cross (32), followed by plating of cross progeny on JL2497 and screening for the indicated phenotype; in each case, the *c*I-*cro* interval was sequenced to verify the presence of the desired mutations. λJL506: λJL163 was crossed with plasmid pLR1 *c*I Y210N; cells were treated before infection with UV irradiation to activate RecA, leading to CI cleavage and loss of immunity, allowing phage growth. Clear plaques were analyzed. λJL514: λJL163 was crossed with pJWL505; slightly turbid plaques were analyzed. λJL515: λJL514 was crossed with pJWL244 (32); clear plaques were analyzed. λJL516: λJL506 was crossed with pJWL506; turbid plaques were analyzed. λJL520: λJL515 was crossed with pJWL506; turbid plaques were analyzed.

Isolation of cI Y210N Lysogenizing Variants. Spontaneously arising lysogenizing variants of λ *c*I Y210N were isolated as a pool by infecting the *hfl*$^-$ strain JL6039, which shows a high frequency of lysogenization after single infection, at a multiplicity of ≈1 with several independent stocks of λJL506, followed by growth overnight in the presence of kanamycin, yielding a pool of lysogens. To enrich for those carrying phages that could undergo prophage induction, these cultures were diluted, grown to exponential phase, and irradiated at 10, 20, and 40 J/m^2. Turbid plaques were purified, and lysogens were characterized.

Physiological Tests. Prophage induction was performed as described in ref. 22. Lysogenization frequencies were determined as described in ref. 32, except that 300 μl LB was added to the top agar. Burst sizes were measured as described in ref. 32, and the average of two experiments is given in the text. Lysogen tests were performed as described in ref. 36. For unstable lysogens, lysogens were first transformed with pFG600 (35), which overexpresses λ CI.

Isolation and Biochemical Characterization of CI Proteins. Wild-type and mutant CI proteins were expressed from T7p::*c*I fusions and purified by extensive modifications of the method of Johnson *et al.* (37), as described in the SI. Cooperativity was measured by using quantitative DNase I footprinting to measure affinities given by the concentration of dimers giving half-maximal protection. Footprinting was carried out as described in ref. 38, by using templates labeled as described in ref. 39 under reaction conditions used by Burz and Ackers (17). Details are given in the SI. The cooperativity coefficient, ω, is given (38) by K_1K_2/R_1R_2, where K_1 and K_2 are the intrinsic affinities of dimers (expressed as dissociation constants) for O_R1 and O_R2, respectively, and R_1 and R_2 are the apparent affinities for the respective sites measured on a template with both operators. With small cooperativity parameters, if $K_1 \ll K_2$, as in the λ case, $K_1 \approx R_1$, and $\omega \approx K_2/R_2$. This value of ω was measured as 1.4 and 2.3 for CI Y210N and CI Y210H, respectively (Fig. 2). RecA-mediated cleavage reactions were carried out as described in ref. 40.

ELISA. Lysogenic and nonlysogenic cultures were grown for several generations to 2×10^8 cells per ml. Cells were recovered by centrifugation, and protein was extracted by using the BugBuster HT Protein Extraction Reagent (Novagen). Relative protein concentrations were determined by using the Bio-Rad protein assay reagent. The ELISA was carried out by using affinity-purified rabbit anti-CI antibody as described in ref. 41 with minor modifications in washing steps. Equal amounts of protein were added to each well; extracts of lysogens were mixed in various proportions with that from a nonlysogen to make a standard curve. HRP-labeled goat anti-rabbit secondary (Bio-Rad) was used and visualized by using the Amersham ImmunoPure TMB substrate kit (Amerhsham, Piscataway, NJ); plates were analyzed on the Molecular Devices (Sunnyvale, CA) Spectra Max 340PC plate reader.

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1. Ptashne M (2004) *A Genetic Switch: Phage Lambda Revisited* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
2. Little JW, Mount DW (1982) *Cell* 29:11–22.
3. Roberts JW, Devoret R (1983) in *Lambda II*, eds Hendrix RW, Roberts JW, Stahl FW, Weisberg RA (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 123–144.
4. Little JW (1993) *J Bacteriol* 175:4943–4950.
5. Johnson AD, Meyer BJ, Ptashne M (1979) *Proc Natl Acad Sci USA* 76:5061–5065.
6. Koblan KS, Ackers GK (1991) *Biochemistry* 30:7822–7827.
7. Révet B, Von Wilcken-Bergmann B, Bessert H, Barker A, Müller-Hill B (1999) *Curr Biol* 9:151–154.
8. Dodd IB, Shearwin KE, Perkins AJ, Burr T, Hochschild A, Egan JB (2004) *Genes Dev* 18:344–354.
9. Dodd IB, Perkins AJ, Tsemitsidis D, Egan JB (2001) *Genes Dev* 15:3013–3022.
10. Johnson AD, Poteete AR, Lauer G, Sauer RT, Ackers GK, Ptashne M (1981) *Nature* 294:217–223.
11. Shea MA, Ackers GK (1985) *J Mol Biol* 181:211–230.
12. Koblan KS, Ackers GK (1991) *Biochemistry* 30:7817–7821.
13. Hochschild A, Ptashne M (1986) *Cell* 44:681–687.
14. Whipple FW, Kuldell NH, Cheatham LA, Hochschild A (1994) *Genes Dev* 8:1212–1223.
15. Benson N, Adams C, Youderian P (1994) *Mol Microbiol* 11:567–579.
16. Beckett D, Burz DS, Ackers GK, Sauer RT (1993) *Biochemistry* 32:9073–9079.
17. Burz DS, Ackers GK (1994) *Biochemistry* 33:8406–8416.
18. Bell CE, Frescura P, Hochschild A, Lewis M (2000) *Cell* 101:801–811.
19. Luo Y, Pfuetzner RA, Mosimann S, Paetzel M, Frey EA, Cherney M, Kim B, Little JW, Strynadka NCJ (2001) *Cell* 106:585–594.
20. Meyer BJ, Maurer R, Ptashne M (1980) *J Mol Biol* 139:163–194.
21. Sarai A, Takeda Y (1989) *Proc Natl Acad Sci USA* 86:6513–6517.
22. Michalowski CB, Short MD, Little JW (2004) *J Bacteriol* 186:7988–7999.
23. Michalowski CB, Little JW (2005) *J Bacteriol* 187:6430–6442.

24. Reichardt L, Kaiser AD (1971) *Proc Natl Acad Sci USA* 68:2185–2189.
25. Shih MC, Gussin GN (1984) *Proc Natl Acad Sci USA* 81:6432–6436.
26. Ferrell JE, Jr (2002) *Curr Opin Cell Biol* 14:140–148.
27. Little JW (1983) *J Mol Biol* 167:791–808.
28. Bailone A, Levine A, Devoret R (1979) *J Mol Biol* 131:553–572.
29. Burz DS, Beckett D, Benson N, Ackers GK (1994) *Biochemistry* 33:8399–8405.
30. Koudelka AP, Hufnagel LA, Koudelka GB (2004) *J Bacteriol* 186:7659–7669.
31. Whipple FW, Hou EF, Hochschild A (1998) *Genes Dev* 12:2791–2802.
32. Little JW, Shepley DP, Wert DW (1999) *EMBO J* 18:4299–4307.
33. Mao C, Little JW (1998) *J Mol Biol* 279:31–48.
34. Little JW, Gottesman M (1971) in *The Bacteriophage Lambda*, ed Hershey AD (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 371–394.
35. Mustard JA, Little JW (2000) *J Bacteriol* 182:1659–1670.
36. Powell BS, Rivas MP, Court DL, Nakamura Y, Turnbough CL, Jr (1994) *Nucleic Acids Res* 22:5765–5766.
37. Johnson AD, Pabo CO, Sauer RT (1980) *Methods Enzymol* 65:839–856.
38. Carlson NG, Little JW (1993) *J Mol Biol* 230:1108–1130.
39. Liu Z, Little JW (1998) *J Mol Biol* 278:331–338.
40. Little JW, Kim B, Roland KL, Smith MH, Lin L-L, Slilaty SN (1994) *Methods Enzymol* 244:266–284.
41. Harlow E, Lane D (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).