JOURNAL OF BACTERIOLOGY, Nov. 2010, p. 6064–6076 Vol. 192, No. 22
0021-9193/10/$12.00 doi:10.1128/JB.00726-10

Stability and Instability in the Lysogenic State of Phage Lambda▽§

John W. Little[1,2]* and Christine B. Michalowski[1]

Departments of Molecular and Cellular Biology[1] and of Chemistry and Biochemistry,[2] University of Arizona, Tucson, Arizona 85721

Received 22 June 2010/Accepted 11 September 2010

Complex gene regulatory circuits exhibit emergent properties that are difficult to predict from the behavior of the components. One such property is the stability of regulatory states. Here we analyze the stability of the lysogenic state of phage λ. In this state, the virus maintains a stable association with the host, and the lytic functions of the virus are repressed by the viral CI repressor. This state readily switches to the lytic pathway when the host SOS system is induced. A low level of SOS-dependent switching occurs without an overt stimulus. We found that the intrinsic rate of switching to the lytic pathway, measured in a host lacking the SOS response, was almost undetectably low, probably less than 10^{-8}/generation. We surmise that this low rate has not been selected directly during evolution but results from optimizing the rate of switching in a wild-type host over the natural range of SOS-inducing conditions. We also analyzed a mutant, λ*prm240*, in which the promoter controlling CI expression was weakened, rendering lysogens unstable. Strikingly, the intrinsic stability of λ*prm240* lysogens depended markedly on the growth conditions; lysogens grown in minimal medium were nearly stable but switched at high rates when grown in rich medium. These effects on stability likely reflect corresponding effects on the strength of the *prm240* promoter, measured in an uncoupled assay system. Several derivatives of λ*prm240* with altered stabilities were characterized. This mutant and its derivatives afford a model system for further analysis of stability.

Gene regulation often involves complex interlocking circuits whose parts work together to produce systems with particular properties. Most current descriptions of complex gene regulatory circuits are essentially qualitative and represent the causal connections among the components in a wiring diagram. However, complex circuits have "emergent" or systems properties that arise from the interactions of the components. The properties of the parts cannot readily predict these systems properties, and while the wiring diagram may predict the presence of systems properties, it generally does not predict the quantitative behavior of the system. Hence, a combination of quantitative modeling and experimental analysis of systems behavior is needed to provide a deeper understanding of emergent properties.

One important systems property is stability—the ability of a system in a particular regulatory state to maintain that state in the face of noise, such as chance fluctuations in the concentrations of regulatory molecules. A stable system rarely changes state; an unstable one switches readily. In metazoans, many regulatory states, once chosen, are stabilized by mechanisms involving chromatin structure and are essentially irreversible, leading to stable specification of cell type. In prokaryotes, by contrast, most regulatory states appear to be reversible, in keeping with the organism's responsiveness to its environment, and the stability of regulatory states depends on the ongoing function of the regulatory molecules themselves. It is not feasible to predict the stability of a circuit from its wiring diagram, since this lacks details such as the strength of interaction parameters. In addition, state switching is believed in the system we study to involve stochastic features of the system that occur in a subpopulation of the cells (5); such features are not included in a wiring diagram.

In this work, we have analyzed stability in the well-studied phage λ system. Like most viruses, λ can grow and produce new virions, following a developmental pathway termed the lytic pathway. Infected cells express a set of lytic genes, resulting in DNA replication, virion production, and cell lysis after ~60 min. The λ regulatory circuitry can also exist in a highly stable regulatory state, the lysogenic state, in which the viral genome is inserted into the genome of the bacterial host and phage lytic genes are repressed by the action of the CI repressor. This state is highly stable in the absence of perturbations, but lysogenic cells can undergo an epigenetic switch to the lytic pathway. When the host SOS system is induced by DNA damage or treatments that inhibit DNA replication, the host RecA protein is activated to a form that stimulates specific cleavage and inactivation of CI (34, 36). Accordingly, the λ system is balanced such that, during normal growth, the lysogenic state is almost completely stable, but it can be almost completely destabilized by an active mechanism for inactivation of CI.

One contribution to this wide range in stability is the pattern of CI regulation in the lysogenic state. The *c*I gene is expressed under the control of the P_{RM} promoter (Fig. 1A and B), which is regulated in a complex manner in response to CI levels. P_{RM} is intrinsically a very weak promoter but is subject to two forms of positive autoregulation. At low to moderate CI levels (Fig. 1C), CI stimulates P_{RM} ~10-fold by binding to O_R2 and contacting RNA polymerase. Binding to O_R1 and O_R2 is cooperative; occupancy by CI changes over a narrow range of CI concentrations. P_{RM} can be further stimulated about 2-fold when a CI-mediated loop is formed between O_L and O_R (Fig.

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, 1007 Lowell Street, Tucson, AZ 85721. Phone: (520) 621-5629. Fax: (520) 621-3709. E-mail: jlittle@u.arizona.edu.

§ Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 24 September 2010.

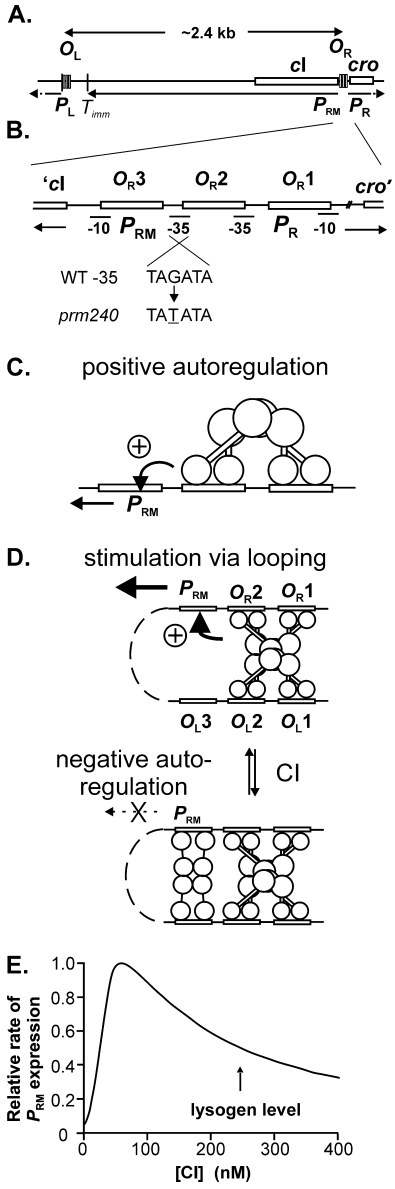

FIG. 1. Maps of λ, functions of CI, and regulation of CI expression. (A) Map of the immunity region of λ, to scale. P_L and P_R transcripts continue beyond the region shown. (B) Expanded map, to scale, of the O_R region, showing the location of the CI and Cro binding sites O_R1, O_R2, and O_R3, the location of the P_{RM} and P_R promoters, and the sequence change in *prm240*. (C) Positive autoregulation of P_{RM} by CI bound at O_R2; CI binds cooperatively to O_R1 and O_R2. Both subunits of a dimer contact a subunit in the other dimer (59), as schematized. (D) Positive and negative autoregulation of P_{RM} by CI-mediated looping. Details of protein-protein interactions in looped forms are not known. (E) Expression of P_{RM} as a function of CI. This curve is the output of a stochastic simulation (J. W. Little and A. P. Arkin, unpublished data), based on a mechanistic model that includes stimulation of P_{RM} by looping. It is meant to illustrate the response of P_{RM} to CI levels, not to represent exact values. It is qualitatively similar to one measured (17) with a reporter construct that did not allow stimulation of P_{RM} by looping and to a curve calculated (1) from a model that includes this feature.

1D), provided that CI is not bound to O_R3 (1, 2). At higher CI levels, CI negatively autoregulates P_{RM} by binding to O_R3 (Fig. 1D), and this binding is favored at lysogenic CI levels by cooperativity due to looping (17). As a result, the rate of CI expression varies markedly in response to CI levels (Fig. 1E). An additional regulatory control is that, at low CI levels, expression from the P_R promoter (Fig. 1A) leads to production of another repressor, Cro protein. Cro then represses P_{RM} by binding to O_R3. This double-negative effect is not essential for switching, but it apparently acts in cells that are "on the edge" to drive them toward switching (3, 54).

Negative autoregulation contributes to the intrinsic stability of the lysogenic state (17). P_{RM} expression increases if the CI level falls, counterbalancing the depletion (9). However, if CI levels fall below a certain level (the peak in Fig. 1E), negative autoregulation no longer occurs. At still lower CI levels, positive feedback begins to fail; this helps drive the system toward switching (19), and synthesis of Cro helps make the switch irreversible. Hence, as CI levels fall to a critical value, which we shall term the "switching threshold," switching becomes increasingly likely. The value for the switching threshold is not known, but measurements of CI activity after SOS induction suggest that it is in the range of 10 to 20% of the lysogen level of CI. This value represents only 25 to 50 monomers of CI in the cell. At such a low value, stochastic events almost certainly play a major role in determining whether switching will occur. Accordingly, two cells with the same history and amount of CI may follow different fates, and the switching threshold should not have a discrete value but a narrow range.

A lysogenic cell that switches to the lytic state after SOS induction produces ~100 virions and releases them into the medium upon lysis. Hence, measurement of free phage in cultures provides a simple assay for switching and an estimate of its rate. Even in an untreated wild-type host lysogenic for λ, the lysogenic state breaks down at an easily detectable level, yielding free phage; about 1 cell in 10^5 switches state (37). This process is termed "spontaneous induction," since it occurs without an overt stimulus and yet depends on the SOS regulatory system, as shown by two lines of evidence. First, host *recA* mutants cannot support cleavage of CI, and *recA* mutant lysogens yield few free phage (10). Second, *c*I *ind* mutations make CI resistant to cleavage; λ*ind* mutant lysogens also yield few free phage (23, 44). A likely explanation for the SOS dependence of free phage release is that cells often undergo sporadic DNA damage, which can induce the SOS system (14). Single-cell experiments show that in a small fraction of untreated cells, the SOS system is induced (40, 47), presumably due to sporadic damage, and it is plausible that in a smaller fraction this induction is severe and prolonged enough to result in prophage induction. These genetic data indicate that spontaneous SOS induction limits the stability of the lysogenic state.

Here we analyze the intrinsic stability of the lysogenic state in the absence of the SOS system. Spontaneous switching of this state presumably follows a pathway similar to that occurring after prophage induction, with the major difference that the latter process includes a mechanism for active CI degradation, whereas spontaneous switching presumably occurs as a consequence of random fluctuations in CI levels. To determine the intrinsic stability of the lysogenic state, we have analyzed stability in a *recA* mutant host, addressing several related questions. First, how stable is this regulatory circuit? As we show, it is extremely stable. Second, what mechanisms contribute to this high degree of stability? Third, is stability dependent on

conditions? Insight into the latter question came from analysis of a mutant, λ*prm240*, whose lysogenic state was only marginally stable. Finally, what is the level of CI at the switching threshold?

MATERIALS AND METHODS

Media, chemicals, and reagents. LB and M9 minimal media are as described previously (43). LBGM, LBMM, and TMG are as described previously (37). Restriction enzymes and DNA ligase were from New England Biolabs or Fermentas Inc. IPTG and XGal were from GoldBioTech. *Pfu* Turbo DNA polymerase for site-directed mutagenesis (22) was from Stratagene; GoTaq master mix (Promega) was used for routine PCR. Polymyxin B was from Sigma. BugBuster HT and rLysozyme were from Novagen.

Bacterial and phage strains. Many of the strains used are listed in Table 1. Construction of phage strains is described in the supplemental material. All bacterial strains were derivatives of *Escherichia coli* K-12. Bacterial strains not listed include those lysogenized with reporter gene fusions and several lysogens not analyzed in detail. All λ derivatives carried the *bor*::*kan* substitution (37), which is not listed. Phage strains isolated as free phage from JL5904 are listed in Table S3 in the supplemental material.

Plasmids. Many of the plasmids used are listed in Table 1; others are listed in the supplemental material, which describes most of the constructions as well.

Phage methodology. General phage methodology was as described previously (37). The test for single lysogens was as described previously (48). It was modified for use in strains with an unstable lysogenic state, as follows. Strain JL7198 was infected with M13 KO7 as described previously (62), and the resulting lysate, containing a preponderance of packaged pJWL1063, was sterilized by heating at 65°C for 20 min. It was then used to transduce this plasmid into the strain being tested. Cells were grown to ~10^8/ml, infected with the lysate, and shaken 30 min. To select for cells bearing pJWL1063, cells were spread on ampicillin plates for isolation of single colonies, or ampicillin was added to 100 μg/ml and cells were grown overnight. The test for single lysogens (48) was carried out on colonies or overnight cultures as described. High levels of CI made by pJWL1063 prevent spontaneous phage induction, which interferes with this test. Use of this method requires that the host carry an F plasmid, as JL5902 and JL2497 do.

Single-lysogen test of λ*prm240* lysogens. λ*prm240* was plated on JL2497 (grown on LBMM) at 30°C on M9 glucose plates. Single plaques were streaked on M9 glucose-Kan plates at 30°C. Nineteen single colonies were purified under the same conditions; colonies were then streaked onto tryptone-Kan plates at 37°C to determine colony phenotype and separately transformed with pJWL1063; transformed (Amp^R) colonies were subjected to the single-lysogen test. For each of the three isolates giving medium-sized colonies with a few small ones, 13 transformants were tested, and all were multiple lysogens. The remaining 16 colonies formed small colonies and were single lysogens.

Isolation of free phage. For free phage from lysogens of λJL163, strain JL5904 was used for cultures grown in LBGM or M9 glucose medium; for growth in M9 glycerol or M9 succinate, strain JL6093 was used to prevent adsorption of free phage. Colonies of JL5904 or JL6093 were formed on a tryptone plate at 37°C and inoculated into LBGM. Cultures were grown at 37°C to 5×10^7 to 10×10^7 cells/ml and chilled; cells were removed by centrifugation, and the supernatant fluid was treated with chloroform. Chloroform was removed by adding fluid to a Falcon polystyrene tube, which adsorbs $CHCl_3$, and aliquots were plated as indicated. To distinguish λ*prm240* from the wild type, free phage were plated on JL2497 on plates containing mitomycin C at 0.25 μg/ml in the bottom agar; this low level of mitomycin C allows the indicator to survive. Lysogens of relatively stable strains were grown and treated as described above; lysogens of phages carrying *prm240* were streaked onto M9 glucose or tryptone plates and incubated at 30°C to avoid selective pressure, followed by growth under the conditions indicated.

Sequencing of free phage. DNA sequencing was carried out as described previously (37). Templates were made by PCR of cored plaques, using the appropriate primers. Six isolates (four from cells grown in LBGM and two from cells grown in M9 succinate) for which no mutation was found in the *c*I-*cro* interval were sequenced in the intervals lying between the following coordinates in the λ genome (GenBank accession number J02459.1): left of *sib* to right of *xis*, 27451 to 29250; middle of *gam* to right of *c*III, 32880 to 33550; left of *N* to first part of *O*, 34500 to 39170; no mutations were found in these intervals.

β-Galactosidase assays. β-Galactosidase assays were done as described previously (41), except that assays were done at 37°C to increase the sensitivity. Accordingly, values for P_{RM} are higher than reported (41, 42).

Medium-shift experiments. Cells were grown with aeration in M9 glucose medium at 30°C to 10^8 cells/ml. Cultures were diluted at least 20-fold into LBGM at 37°C and shaken at 37°C. At intervals, the optical density at 590 nm (OD_{590}) was read, and samples were taken for measurement of free phage: an aliquot was centrifuged 1 min at ≥10,000 × *g*, and the supernatant fluid was treated with chloroform. In some experiments, aliquots were also taken to measure CI levels: a portion (generally 2 optical density units [ODU]) of the culture was chilled rapidly to 0°C, followed by centrifugation 1 min at ≥10,000 × *g*; pellets were resuspended in 1 ml 10 mM Tris-HCl (pH 8)–10 mM $MgSO_4$ and centrifuged 1 min at ≥10,000 × *g*; pellets were frozen at −20°C for processing as indicated below. In some experiments, several different dilutions of the initial culture were made to allow OD_{590} measurement over a longer time frame.

Measurement of CI levels. CI levels were determined using Western blots, using a rabbit polyclonal antibody. Cells isolated as described above were lysed using BugBuster HT (50 μl per ODU of cells) containing rLysozyme. Protein concentrations were measured using the Bio-Rad protein assay reagent. Samples for SDS gel electrophoresis contained a constant amount of protein per well; in some cases, extracts were diluted with an extract from a nonlysogenic strain grown under the same conditions as the sample being assayed. JL6112 extracts were compared with JL5904 extracts diluted with JL5902 extract. Following electrophoresis, protein was transferred to a nitrocellulose membrane (Schleicher and Schuell BA83), blocked, and exposed to rabbit polyclonal antibody against CI, followed by goat anti-rabbit secondary antibody conjugated with IRDye 800CW (LI-COR). Dried membranes were scanned using the LI-COR Odyssey infrared imaging system. A gel mobility shift assay, done as described previously (17), also gave results consistent with the those of the Western blotting experiment for strain JL6112 grown in LB medium (not shown).

RESULTS

Stability of the lysogenic state. To measure the frequency with which the lysogenic state switches to the lytic state, we grew cultures of the Δ*recA* (λ^+) strain JL5904 to mid-exponential phase, removed the cells by centrifugation, and characterized phage in the supernatant fluid. Some phage form clear plaques and carry *c*I mutations; others form turbid plaques, because lysogens can form in the center of the plaque. We analyzed free phage forming turbid plaques. The prophage carried a marker for kanamycin resistance, allowing selection for lysogens by growth on kanamycin plates. Adsorption of released phage to the cells was prevented by growth in media containing glucose, which represses the LamB receptor to which λ binds (29, 61).

We reported (37) that such cultures contain ~500 free phage/ml that form turbid plaques. Others have obtained similar results (5, 10, 51). We later found that this value is variable (see below). In addition, two lines of evidence showed that almost all free phage forming turbid plaques were not wild-type λ, but mutants. First, lysogens arising in most turbid plaques formed a range of colony sizes, while lysogens of λ^+ formed uniformly large colonies. For reasons given below, we term this the "unstable lysogen phenotype." Second, and strikingly, sequencing of the *c*I-*cro* interval from many isolates with this phenotype showed that all had the same mutation in the P_{RM} promoter, which we term *prm240* (Fig. 1B). This mutant was isolated from all cultures analyzed; it is described below ("Analysis of λ*prm240*"). We conclude that most of the free phage forming turbid plaques are mutants, a conclusion also reached on the basis of other evidence by others (7).

To measure the titer of wild-type free phage, we used a screen to discriminate phage with *prm240* from wild-type phage. Phage were plated on a *recA*$^+$ indicator in the presence of the SOS-inducing agent mitomycin C; λ^+ formed faintly turbid plaques, while λ*prm240* formed clear plaques. Without mitomycin C, the titer of turbid plaques varied substantially, as

TABLE 1. Strains employed

Strain, phage, or plasmid	Relevant genotype	Source or reference
Bacterial strains		
JL2497	N99 *lacZ*ΔM15/F′ *lacI*^{q} *lacZ*ΔM15::Tn*9;* used as wild type	37
JL5016	JL5902 (λJL615)	This work[a]
JL5024	JL6112 resistant to λ	This work[c]
JL5902	JL2497 Δ(*srl-recA*)306::Tn*10*	37
JL5904[b]	JL5902 (λJL163)	37
JL5906[b]	JL5902 (λJL169)	37
JL5908[b]	JL5902 (λJL172)	37
JL5924[b]	JL5902 (λJL175)	37
JL5932[b]	JL2497 (λJL163)	37
JL6093	JL5904 resistant to λ	This work[c]
JL6112	JL5902 (λJL240)	This work[a]
JL6142	JL2497 Δ(lacIPOZYA) F$^-$	4
JL6799	JL2497 (λJL240)	This work[a]
JL6839	JL2497 (λJL615)	This work[a]
JL6994	JL6142/pJWL615/pJWL486	41
JL6995	JL6142/pJWL615/pA3B2	41
JL7198	JL468/pJWL1063	This work
Phage strains		
λJL163	λ$^+$ *bor*::*kan*, used as wild type	37
λJL169	λ O_R121	37
λJL172	λ O_R3′23′	37
λJL175	λ O_R323	37
λJL176	λ *v3* (mutation in O_R1)	This work[d]
λJL188	λ O_R323 derivative with O_R1 sequence TA<u>T</u>C<u>C</u>CT<u>T</u>GCGGT**<u>A</u>**ATA[e]	37
λJL240	λ*prm240*	This work
λJL291	λ O_R2: TAACACCGT<u>C</u>CGTGTTG	This work[f]
λJL293	λ O_R2: TAACACC<u>A</u>TGCGTGTTG	This work[f]
λJL343	λ O_R1: TA<u>A</u>CTCTGGCGGTGATA	This work[f]
λJL465	λ*prm240* O_R2^-	This work[d]
λJL466	λ*prm240* O_R1^-	This work[d]
λJL473	λ*prm240* O_L3-4	This work[d]
λJL615	λ*prm240 cro-z8*	This work[d]
λJL815	λ *c*I D38N	41
λJL1387	λ*prm240* O_R3-*r1*	This work[d]
M13 KO7	Helper phage for packaging pJWL1063	62
Plasmids		
pA3B2	Δ35 *lacP*::*c*I; provides low level of CI; Cm^r; vector pACYC184	63
pGB2	Spc^r; compatible with pACYC184 and ColE1-derived plasmids	12
pJWL334	Modified polylinker; high copy no.; vector pBS(−)	42
pJWL486	Vector control for pA3B2; vector pACYC184	42
pJWL615	*lacI*^{q}; vector pGB2	42
pJWL1063	*lacP*::*c*I *oriM13*; vector pBR322	This work[d]
pRS414, pRS591	Vectors for making *lacZ* protein fusions; vector pBR322	56
pRS1274	Vector for making *lacZ* operon fusions (described as pRS528 in reference 56); vector pBR322	56

[a] Lysogens of λJL240, λJL473, λJL615, and λJL1387 were isolated by plating phage on tryptone plates at 30°C, followed by selection for lysogens by streaking on tryptone plates with kanamycin at 30°C and a single lysogen test (see Materials and Methods). Lysogens of λJL465 and λJL466 were isolated in the same way but on M9 glucose plates at 30°C.

[b] Lysogen was used and described in reference 37 but not identified by strain number.

[c] JL6093 and JL5024 were isolated from JL5904 and JL6112, respectively, by selection for resistance to λ*vir* (from D. Mount). These strains cannot adsorb λ.

[d] Isolated as described in the supplemental material.

[e] The altered O_R1 sequence in λJL188 was derived (37) from λJL175, which contains three changes (underlined) in O_R1, by a fourth mutation, shown in bold, changing G to A. This latter mutation probably weakens binding of Cro to the operator, based on the way it was isolated, and probably CI binding as well; it also likely weakens P_R, since it changes the first base of the −10 region away from consensus. This phage exhibited the unstable lysogen phenotype in the Δ*recA* host, with a growth defect on tryptone plates more severe than that of λ*prm240* lysogens at 37°C. Colony size on minimal plates was like that of the wild type at both temperatures.

[f] Isolated as described in the text and in Table S3 in the supplemental material. The mutated bases are underlined; the corresponding WT bases for the mutations in λJL291, λJL293, and λJL343 are G, G, and C, respectively. The O_R1 mutation was reported to weaken CI binding by only ~0.6 kcal/mol (52), a small increase whose effect should be even less due to pairwise cooperative DNA binding. Hence this mutation might have little effect on stability. The effect of the O_R2 mutations on CI binding has not been tested. These mutants were isolated in screens that did not involve the use of mitomycin C.

expected if most arose by mutation (Table 2). With mitomycin C, most plaques were clear. Phage from turbid plaques were isolated, and the *c*I gene and the O_R region were sequenced (Table 2). For isolates with no mutations, the O_L region was also sequenced. In total, 42 isolates with no mutations in these intervals were isolated from 12 ml of culture supernatant.

Other screens also yielded isolates for which no mutations were found. For several of these we sequenced other intervals

TABLE 2. Analysis of free phage from a Δ*recA* (λ^+) mutant lysogen[a]

Culture	No. of free phage/0.6 ml without mitomycin C		No. of turbid plaques/2 ml with mitomycin C	Sequences of turbid plaque isolates
	Clear	Turbid		
A	57	44	10	10 WT
B	102	326	9	4 WT, 5 *c*I S149F
C	104	29	5	5 WT
D	62	34	13	13 WT
E	90	107	15	10 WT, 5 *c*I S149F
F	62	73	0	0
Totals			52/12 ml	42 WT, 10 *c*I S149F/12 ml

[a] Free phage from six independent 50-ml cultures were isolated as described in Materials and Methods and plated on JL2497. Plaques formed without mitomycin C were scored for clear or turbid phenotype. Since most of the phage forming turbid plaques carry *prm240*, the abundance of phage with this allele is about the same as those forming clear plaques due to mutation of the *c*I gene, indicating that *prm240* is a mutational hot spot. High titers of turbid plaque formers in culture B, and perhaps E and F, probably resulted from "jackpots," in which a *prm240* mutant arose early in the culture, as likely also occurred previously (37). For plaques formed with mitomycin C (0.25 μg/ml) in the bottom agar, only turbid plaques were scored. Sequences of the *c*I-*cro* interval and the O_L region were determined. λ*c*I S149F showed the unstable lysogen phenotype. The S149F allele changes the catalytic serine residue involved in CI autocleavage (58); plaques of this phage, which are less turbid than the wild type without mitomycin C, remain turbid in its presence since CI cleavage cannot occur. The mutant protein is somewhat defective for repressor function (63), probably leading to frequent switching. Other *c*I *ind* mutants would also form turbid plaques, but perhaps few such mutants are also leaky for repressor function.

TABLE 3. Free phage levels in unstable lysogens[a]

Phage	Mutation	Free phage level in *recA* lysogen (phage/ml)
λJL176[b]	O_R1 *v3*	1,000
λJL815[c]	*c*I D38N	4×10^5
λJL169[d]	O_R121	6,000
λJL172[d]	$O_R3'23'$	500
λJL175[d]	O_R323	10^5
λJL163	None (WT)	(2 to 5)

[a] Cells were grown and free phage were recovered as described in Materials and Methods. The titer of turbid plaques is indicated. The value for the WT, from Table 2, is that of WT free phage only and is given for comparison.

[b] Lysogens of λ*v3* formed colonies of normal size. The value given for free phage titer was about the same in six independent cultures in the same experiment. Of 24 plaques tested, all gave uniformly large Kan^r colonies. Of six isolates sequenced in the O_R region, all retained *v3* and had no other changes.

[c] Of seven free phage sequenced in the *c*I-*cro* interval, all had the *c*I D38N mutation and O_R^+.

[d] Free phage forming turbid plaques from the λO_R323 lysogen were probably of that genotype, since they were present at high levels and formed the small plaques characteristic of λO_R323. Lysogens of λO_R121 and λ$O_R3'23'$ yielded the given titer of free phage forming turbid plaques in each of three independent cultures. We sequenced six isolates of each; in each case, all six had the parental sequence in the *c*I-*cro* interval; in the O_L region, all were wild type, except that one derivative of λ$O_R3'23'$ had a mutation in O_L1 that should weaken CI binding substantially.

involved in gene regulation (see Materials and Methods), and found no mutations, suggesting that they are indeed wild type. We conclude that wild-type free phage are present at roughly 2 to 5 phage/ml culture. We calculate (see Discussion) that the frequency of switching is probably $<10^{-8}$ per cell per generation.

We speculated that a wild-type lysogen might be less stable during growth in minimal medium; slowly growing cells are smaller, so that stochastic effects on *c*I expression or function might be more frequent. To test this idea, we grew JL5904 or JL6093 in minimal medium with glucose, glycerol, or succinate as the carbon source. Contrary to our expectation, the free phage titer was very low. This resulted in part because λ virions are somewhat unstable in minimal medium, with a half-life of 30 to 60 min (not shown). Despite this complication, we infer that the low titer also reflected a low rate of free phage production. Few of the isolates conferred the unstable lysogen phenotype. They included *c*I mutants (see Table S3 in the supplemental material) and an O_R1 mutant, as well as several for which no mutations could be found. The instability of λ virions prevents a quantitative estimate of the titer of wild-type phages, but we may conclude that growth in minimal medium does not markedly destabilize the lysogenic state.

Analysis of mutant free phage and existing mutants affecting stability. In addition to wild-type phage, in the above screens we recovered free phage with mutations in *c*I or in O_R (Table 1; see Table S3 in the supplemental material). We analyzed free phage levels from lysogens of these mutants. Surprisingly, lysogens of λJL291, λJL293, and λJL343, with changes in O_R2 or O_R1, did not release markedly elevated levels of free phage (data not shown). It is uncertain whether the level is higher than that of the wild type and unclear why these mutants were initially recovered as free phage. Several mutants with changes in *c*I gave a range of phenotypes, and lysogens in JL5902 had a wide range of stabilities (see Table S3 in the supplemental material).

We also analyzed several existing mutants expected to have a lower level of CI and/or a higher switching threshold, and found (Table 3) that each had a substantially higher switching rate than the wild type. The first has an O_R1 mutation, *v3*, which weakens binding of CI to O_R1 but has almost no effect on Cro binding (52, 60). We attribute the higher level of free phage to the expectation that, in a *v3* lysogen, the occupancy of O_R1 and O_R2 by CI would be lower than in a λ^+ lysogen and hence that its switching threshold would be elevated. The second mutant carries the *c*I D38N mutation, which nearly abolishes positive autoregulation of *c*I from P_{RM} (31, 41) and should reduce the level of CI; it also markedly destabilized the lysogenic state.

In the third type of mutant tested, O_R1 and/or O_R3 are mutated so that both have the same sequence (37). In λO_R121, O_R3 is changed to the sequence of O_R1; in λO_R323, O_R1 is changed to O_R3; and in λ$O_R3'23'$, both sites are changed to a hybrid site termed O_R3'. Consistent with previous findings (37), the present, more-detailed analysis showed that all three mutants were less stable than the wild type. We ascribe this to the reduced level of CI found in mutant lysogens (37). We previously suggested that the CI level is reduced because CI occupancy patterns at O_R are altered (37). Subsequent work suggests that these changes might also affect looping between O_L and O_R (1, 17), giving aberrant looped forms that are not generally favored in the wild type. The effect of this is hard to predict: it might reduce CI levels if it prevents looping-mediated activation of P_{RM} (1), this activation might instead be more efficient, or changes in looped forms might affect the cooperative interactions among CI dimers that lead to negative autoregulation.

In sum, our evidence indicates that the stability of the lysogenic state can be reduced by mutations, either in *cis*-acting sites or in CI itself, that weaken the ability of CI to repress the lytic promoters and/or to regulate CI expression. Presumably, the proper choice of mutations in *c*I, in the operators, or in P_{RM} could afford any desired level of stability. We turn next to a mutant that lies at the opposite end of the stability spectrum from the wild type.

Analysis of λ*prm240*. The following evidence indicates that lysogens of λ*prm240* are barely stable and readily switch their regulatory state from lysogenic to lytic. As noted above, λ*prm240* showed the "unstable lysogen phenotype"; among lysogens arising in a plaque, most formed small colonies, while a small fraction made larger colonies. Since *prm240* is a down-mutation in P_{RM} (see below), we surmised that cells in small colonies made a low level of CI and often switched to the lytic state, reducing the number of progeny cells, while cells in the larger colonies made more CI and switched less frequently. We found that lysogens forming small colonies carried a single copy of the prophage, while those making large colonies carried multiple tandem prophages (see Materials and Methods), and presumably made more CI due to gene dosage. Further analysis used single lysogens.

Remarkably, growth conditions influenced the severity of the unstable lysogen phenotype. Colony sizes of *recA*$^+$ (λ*prm240*) and Δ*recA* (λ*prm240*) lysogens were much closer to those of wild-type lysogens at 30°C than at 37°C, suggesting that the lysogenic state was more stable at 30°C. On minimal medium, colony sizes of λ*prm240* and wild-type lysogens were the same, suggesting that λ*prm240* lysogens were relatively stable in minimal medium. The mechanistic basis for these conditional responses is unclear.

These responses to growth conditions were not specific to *prm240*. Other P_{RM} mutants also show the unstable lysogen phenotype (42). We tested six such isolates on the Δ*recA* host. Colonies of lysogens were the same size as wild-type colonies on minimal plates. On tryptone plates, most were like the wild type at 30°C, while MD17 and MD12 were slightly smaller; at 37°C, colony sizes were, in rank order, wild type (WT) > MD12 > MD13 > λ*prm240* ≈ MD8 > MD17 ≥ MD11 ≈ MD47. Hence, the detailed response to growth conditions may depend on the properties of the given promoter.

We analyzed further the stability of λ*prm240* lysogens in a range of growth media, at both 30°C and 37°C. Few free phage were released in minimal medium (Table 4), consistent with the colony phenotype and the paucity of λ*prm240* isolates from JL5904 grown in minimal medium. At higher growth rates, titers of free phage increased markedly. At the highest growth rate, in LB medium at 37°C, cultures grew slowly (see also below) and released very high levels of free phage. We conclude that growth at higher rates markedly destabilizes λ*prm240* lysogens, as does growth at higher temperatures to a lesser extent.

To assess the time course with which the lysogenic state was destabilized after a shift from minimal to rich medium, we followed growth and phage production of *recA*$^+$ and Δ*recA* λ$^+$ and λ*prm240* lysogens after a switch from the most stable to the least stable growth condition. After growth in minimal medium at 30°C, cells were transferred to LB medium at 37°C. In both host strains, λ*prm240* lysogens grew like the wild-type control for about 2 h, followed by growth at a lower rate (Fig. 2B and C). Levels of free phage underwent a progressive and enormous increase with time, reaching a high value at about 3 h, after which the level of free phage increased more slowly as cell growth continued (Fig. 2A).

TABLE 4. Free phage titer of λ*prm240* lysogen[a]

Medium	Temp (°C)	Doubling time[b] for λJL163 lysogen (min)	Free phage/cell[b] for λ *prm240* lysogen	37°C/30°C[c]
M9 glucose	30	160	5.4×10^{-6}	11
	37	110	5.9×10^{-5}	
M9 glucose + Casamino Acids	30	127	0.012	18
	37	73	0.21	
Tryptone broth	30	80	0.08	16
	37	54	1.3	
LB	30	72	1.8	10
	37	44	17	

[a] Strain JL6112 was grown overnight in tryptone broth at 30°C and then grown as follows. For growth in M9 glucose with or without Casamino Acids, an aliquot was grown exponentially for many generations in M9 glucose at 30°C, followed by exponential growth for at least five generations under the indicated conditions. For growth in tryptone or LB, an aliquot was diluted into tryptone and grown for three generations at 30°C, followed by exponential growth for at least five generations in the indicated conditions and sampling for free phage as described in Materials and Methods.

[b] For calculation of doubling time and phage/cell, cell growth and cell numbers were determined on cultures of JL5904 growing in parallel. Titers given for minimal medium are likely underestimated by a factor of 2 to 5, since λ virions were somewhat unstable in minimal medium (see text). To test for readsorption of free phage in tryptone broth (which lacks glucose), in a separate experiment strain JL6112 and its λ-resistant derivative JL5024 were grown in parallel in tryptone broth, at both 30 and 37°C. At each temperature, free phage levels for both cultures were the same within experimental error. Hence, readsorption is not significant under our conditions.

[c] Ratio of titer of free phage at 37°C to that of free phage at 30°C.

The magnitude of these effects was greater in the *recA*$^+$ host than in its Δ*recA* counterpart. The *recA*$^+$ (λ*prm240*) culture underwent partial lysis, followed by an essentially flat growth curve (Fig. 2B). Evidently the likelihood of switching was about the same as that of dividing. Net growth of the *recA* mutant (λ*prm240*) culture slowed, presumably because a substantial fraction of the cells in the culture switched and lysed. Free phage levels in the *recA*$^+$ culture were ~10-fold higher than in its *recA* mutant counterpart. The more frequent switching in *recA*$^+$ cells may result in part from the presence of activated RecA in a fraction of the cells in the culture; the greater yield of free phage may result both from higher switching rates and the generally healthier state of *recA*$^+$ cells.

We reasoned that the CI levels in the λ*prm240* lysogen grown under a range of conditions could provide insight into the level of CI at the switching threshold for λ*prm240*—that is, the level of CI at which switching becomes likely (see introduction). We compared CI levels in the λ$^+$ and λ*prm240* lysogens in cultures grown in tryptone broth at 30° and in LB medium at 37°C using Western blotting to quantify CI levels; the respective values were about 30% and 20%. The precision of this assay was not sufficient to allow a detailed correlation between CI levels and switching rates under various growth conditions. We conclude that the switching threshold for

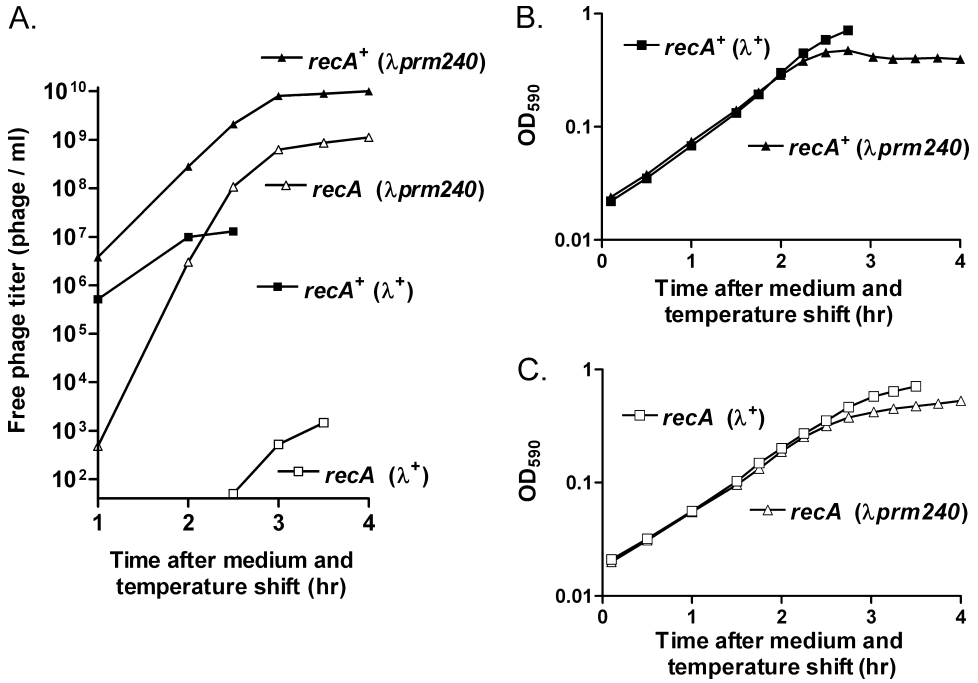

FIG. 2. Destabilization of λ*prm240* lysogens after medium and temperature shift. Cultures were grown and sampled as described in Materials and Methods. All measurements were from the same experiment and are separated for clarity. Strains were JL5932 and JL6799 (*recA*$^+$), and JL5904 and JL6112 (Δ*recA*). (A) Free phage levels for all strains. Note the logarithmic scale. (B and C) Growth curves for *recA*$^+$ (B) and *recA* mutant (C) lysogens. OD_{590}, optical density at 590 nm. Cell debris from lysis of λ*prm240* lysogens made a minor contribution to the OD_{590} at later times.

λ*prm240* is about 25% of the wild-type level of CI (see Discussion).

Mutations affecting stability of λ*prm240*. We analyzed the effects of mutations that stabilized or further destabilized λ*prm240*. We first compared Δ*recA* λ*prm240* lysogens with those of λJL465 and λJL466 (with additional mutations in O_R2 and O_R1, respectively). Under all conditions tested (30 and 37°C in M9 medium and in tryptone broth), titers for λJL465 and λJL466 were elevated about 5- and 10-fold, respectively, relative to those for λ*prm240* (data not shown). In tryptone broth at 37°C, growth of λJL465 and λJL466 lysogens nearly ceased, as seen in LB medium for the *recA*$^+$ (λ*prm240*) lysogen (Fig. 2B), suggesting that the likelihood of switching was about equal to that of cell division. We conclude that the O_R2 and O_R1 mutations in these isolates destabilize λ*prm240*, presumably by weakening binding of CI to these sites. The finding that lysogens of the single O_R1 and O_R2 mutants still had very low levels of free phage (see above) further emphasizes the extreme stability of the wild type.

We next removed or weakened negative autoregulation by combining *prm240* with either of two changes. The first derivative, λJL473, carried the *r1* mutation in O_R3; this mutation eliminates negative autoregulation at CI levels found in a WT lysogen (17). The second derivative, λJL1387, carried O_L3-4, with four mutations in O_L3, abolishing CI binding to O_L3 (18). This change also reduces negative autoregulation (Fig. 1D). We expected with either mutant that, if negative autoregulation was substantial in a λ*prm240* lysogen, then relieving it would lead to higher CI levels in the *prm240* O_R3-*r1* and *prm240* O_L3 mutants, markedly stabilizing the lysogen. We found that yields of free phage after extended growth in LB medium at 30 or 37°C were reduced 5 to 10-fold from that seen with λ*prm240* (data not shown), suggesting that the rate of switching is reduced by 5- to 10-fold in the double mutants. This modest effect suggests that negative autoregulation was scarcely operative during growth in LB medium. Consistent with these reduced switching rates, growth of lysogens in LB medium was only slightly slower than that of the λ$^+$ lysogen (data not shown), in contrast to the marked reduction of growth of the λ*prm240* lysogen (Fig. 2).

It is not clear what the course of events is during the switching process of λ*prm240*. Presumably an early event is transient expression of the lytic promoters P_L and P_R. An early product of P_R expression is the Cro protein, which can act to repress P_{RM}. We reasoned that, if Cro plays an essential role in switching, then removing Cro should stabilize the mutant lysogen. Complete removal of Cro by mutation is lethal for lytic growth (20). However, if P_{RM} repression plays an important role in the switching process, then reducing the level of Cro by mutation might act to stabilize λ*prm240*.

We tested this expectation with the use of a mutation termed *cro-z8*, which changes the 5′-UTR of the *cro* mRNA and gives a reduced level of Cro (46). We (J. W. Little and K. Newell, unpublished data) reisolated this mutation as a suppressor of a lysogenization defect in a mutant phage (see supplemental material), a property suggesting that it has a functional defect in Cro function. As judged by its effect on expression of a P_R::*lacZ* protein fusion, *cro-z8* reduced Cro expression to ~25 to 30% of the wild-type level (Table 5). Hence, the *cro-z8* mutation might be expected to stabilize λ*prm240*. Comparison

TABLE 5. Effect of *cro-z8* mutation on P_R expression and on stability of λ*prm240* under various growth conditions

Medium	Growth temp (°C)	β-Galactosidase activities[a]			Phage yield (phage/cell)[b]		
		P_R^+	P_R^+ *cro-z8*	Ratio	λ*prm240*	λ*prm240 cro-z8*	Ratio
Tryptone	30	2,304	714	0.31	0.08	0.008	10
	37	3,869	1,268	0.33	1.3	0.2	6.5
LB	30	2,203	730	0.33	1.8	0.37	4.8
	37	3,491	872	0.25	17	7	2.3

[a] For the β-galactosidase assays, reporters carried the *prm240* allele and either the wild-type or *cro-z8* mutant allele; the presence of the *prm240* allele made little or no difference in the activity of P_R under these conditions (not shown). We surmise that the effect of the *cro-z8* mutation differed from the lower value (11%) found when Cro was expressed from the *lac* promoter on a plasmid (46) due to differences in the 5′ leader of the mRNA. Since P_R was more active at 37°C than at 30°C, both in tryptone broth and in LB, direct comparisons between results at different temperatures are not meaningful, since the level of Cro should also be somewhat higher in the *cro-z8* lysogen at 37°C.

[b] For measurement of free phage levels, as part of the experiment in Table 4 the λ*prm240 cro-z8* lysogen JL5016 was grown in parallel with JL6112. Data for λ *prm240* phage yields are from Table 4.

of λ*prm240* and λ*prm240 cro-z8* lysogens grown under the same conditions as used for the reporter assays (Table 5) showed that, although *cro-z8* did stabilize the lysogen, the effect was rather modest. As the λ*prm240* lysogen became more unstable, the effect of *cro-z8* became smaller. These data suggest that Cro may play a minor role in certain switching events for λ*prm240* (see Discussion).

Properties of the *prm240* promoter. We assessed the strength and regulation of the *prm240* promoter, using a reporter gene in an uncoupled assay system. The P_{RM}::*lacZ* protein fusions carry either P_{RM}^+ or *prm240* and various alleles of the O_L region distal to the *lacZ* reporter gene (Fig. 3A and supplemental material). The first construct (Fig. 3A, middle) carries O_L and a region lying upstream of O_L3 that supports (1) the stimulatory effect of looping and should mimic the situation in the prophage. We tested its response to graded levels of CI, supplied from a *lacP*::*c*I fusion on a plasmid in the presence of graded levels of IPTG. This plasmid produces some CI in the absence of IPTG. A control strain lacked the CI-producing plasmid. Assays were carried out both in M9 minimal medium and in LB medium at 30°C and at 37°C. Unexpectedly, the shapes of the dose-response curves differed between those for M9 medium and LB medium. This resulted, at least in part, because the levels of CI were much greater in reporter strains grown in M9 medium than in those in LB medium (data not shown; see Fig. 3 legend). Hence, comparisons between the two growth media at a given IPTG level are not meaningful, but comparisons between the WT and *prm240* promoters under a given condition are valid.

Reporter assays (Fig. 3 and Fig. S3 in the supplemental material) showed several notable features. First, *prm240* was somewhat weaker at 37°C than at 30°C (Fig. 3C), suggesting that the CI level would be lower at 37°C, consistent with the higher rate of phage production at 37°C. Wild-type P_{RM} was less responsive to the temperature, showing somewhat higher expression levels at 30°C than at 37°C only at low levels of CI, at which positive autoregulation should be occurring (see Fig. S3 in the supplemental material).

Second, *prm240* was markedly weaker in LB medium than in M9 medium (Fig. 3), both at 30°C and at 37°C. The WT promoter was less affected by the change in growth medium (Fig. S3 in the supplemental material). Though the mechanistic basis for the effects of temperature and growth rate on the strength of *prm240* is not understood, these effects correlate well with the rates of λ*prm240* phage production in response to these changes in conditions.

Third, *prm240* was much weaker than WT under all conditions. In M9 medium at 30°C, its strength was about 20% of that of wild-type P_{RM}. In LB medium, it was much weaker yet. In LB medium, the observed values fit reasonably well with the observed levels of CI in the λ*prm240* lysogen (see Fig. 3 legend).

We next tested whether regulation of *prm240* resembled that of P_{RM}^+. As shown above (Fig. 3), CI stimulates *prm240*, as with the wild type. To test if *prm240* was subject to negative autoregulation and to stimulation by looping, we used reporter constructs (Fig. 3A, top and bottom) with two other alleles of the O_L region—one which has only the T_{imm} terminator and which cannot support looping, and one which has the O_L region but with four mutations in O_L3, abolishing CI binding to that site. For each allele of O_L, reporters with O_R3^+ and the O_R3-*r1* mutation (to reduce negative autoregulation) were made; the assay was done in M9 medium at 30°C to maximize the activity of *prm240*.

In strains with P_{RM}^+, the presence of O_L stimulated expression at low CI levels (Fig. 4A) and led to repression at high levels. Repression was largely prevented in the presence of both of the O_R3-*r1* and O_L3 mutations and weakened by each mutation individually. This pattern, and the magnitude of the stimulation, was expected from previous work (1, 17), although this work did not use an uncoupled assay system in conjunction with the region upstream of O_L as used here.

Strikingly, *prm240* was stimulated by looping to a substantially greater degree (Fig. 4B) than was P_{RM}^+. A large stimulation was also seen in cells grown in LB medium (data not shown), but its magnitude was hard to assess due to the low value in the absence of looping. At higher CI levels, negative autoregulation was observed and was more complete than with WT P_{RM}. This effect was less pronounced when O_R3 or O_L3 was mutated and still less so when both were mutated, as seen with the WT (1), but some residual repression was seen. Hence, though regulation of *prm240* is qualitatively similar to that of P_{RM}^+, quantitative differences are seen (see Discussion).

DISCUSSION

Estimate of switching rate for the WT. We found a low but detectable number of wild-type free phage in cultures of Δ*recA*

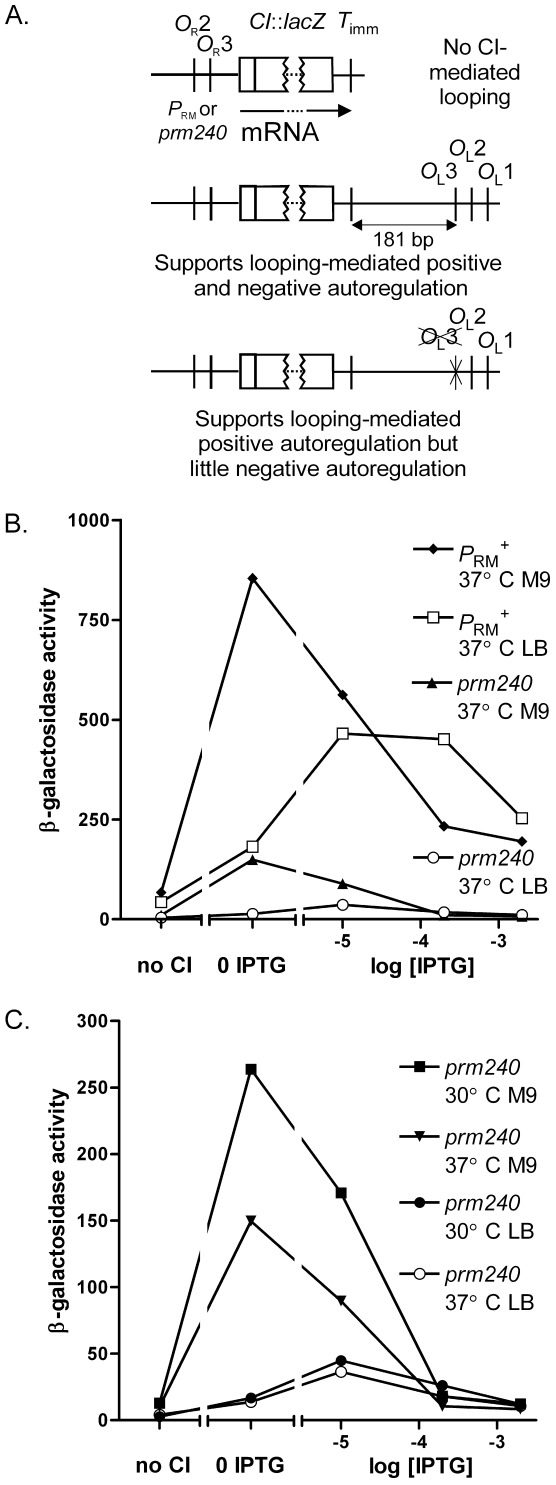

FIG. 3. Reporter constructs and promoter activity for P_{RM}^+ and *prm240*. (A) Structure of reporter constructs (not to scale). The three constructs shown (see the supplemental material) encode an mRNA that is terminated at T_{imm}; hence, all three mRNAs are identical and their levels of expression are directly comparable (1). A second version of each construct contained the *r1* allele in O_R3, which weakens CI binding (see text). Maps are not to scale. (B and C) Reporter assays. Cells were grown at the temperature and in the medium indicated in the figure. Two reporter constructs were used, one with P_{RM}^+ and the other with *prm240* (see text and panel A, middle construct). For each construct and growth condition, two strains were used, one lacking CI and one carrying a plasmid with a weak *lacP*::*c*I fusion. The latter strain was grown with no IPTG or with the indicated levels of IPTG, providing various levels of CI. Data for P_{RM}^+ at 30°C were obtained in separate experiments and are shown in Fig. S3 in the supplemental material. (B). Expression of P_{RM}^+ and *prm240* at 37°C. (C). Data for *prm240* fusions, with an expanded scale. As judged by a gel shift assay (not shown), CI levels in the reporter strain grown in M9 medium at 30°C and in LB medium at 37°C with 2 mM IPTG were roughly 3 times and 1 time, respectively, the level in JL5932 grown in parallel; in LB medium at 37°C, the CI levels in cells grown at 0.01 and 0.2 mM IPTG were roughly 0.1 and 0.5, respectively, of the lysogen level, bracketing the value (~0.2 the lysogen level) seen in JL6112 under these growth conditions. To compare the rates of expression with the levels seen in wild-type and λ*prm240* lysogens, we compared the rate of P_{RM}^+ expression at the wild-type lysogen level (panel B), at 2 mM IPTG, with the rate of *prm240* expression at the CI level in a λ*prm240* lysogen. These values were 250 and ~40 units, respectively; hence, the value for *prm240* is 15 to 20% of the value for P_{RM}^+, similar to the ratio of observed CI levels in lysogens.

(λ^+) lysogens. There are several difficulties in estimating the rate of switching to the lytic pathway. First, *recA* mutant cultures include a sizable fraction of dead cells (11); it is unknown if a cell that cannot form a colony can switch and/or support phage growth. Presumably the number of cells able to switch lies between the viable cell titers measured for this strain and for its *recA*$^+$ counterpart, a value ~2-fold higher in our strains. Second, the burst size of cells that have switched is not known. Possibly it is lower than observed after UV irradiation or infection, since residual CI may partially repress the lytic promoters (54). However, we have found (unpublished data) that in JL5932, the *recA*$^+$ counterpart of JL5904, the burst size of cells given a low UV dose is substantially larger than reported (54), suggesting that the effect of residual CI is not large in our strains, at least after UV induction. Third, the burst size likely varies widely after individual switching events. Wide variability in individual burst sizes has been observed with lytic phages after single (16) or multiple (30) infection and with λ after multiple infection (33); since our estimates involved only a few switching events, this adds another level of uncertainty. Finally, the switching rate is so low that probably only a few events occurred in the cultures we studied, adding some statistical uncertainty.

With these caveats, we can make a rough estimate of switching rates, if we assume that the number of cells that can switch equals the number of viable cells and that the average burst size is 100 phage per switching event. We observed ~4 free wild-type phage/ml culture at 5×10^7 viable cells/ml, or ~2 switching events in a 50-ml culture. These phage represent the progeny of cells that had switched to the lytic state prior to 1 h before, a time at which the cultures contained $\sim 10^9$ cells. Accordingly, of 10^9 cells arising by cell division about two had switched, i.e., there were $\sim 2 \times 10^{-9}$ switching events per cell division. Given all the uncertainties, a conservative estimate is that the switching rate is probably $< 10^{-8}$ per cell division.

This number is so low that its exact value is not very meaningful. It is lower than the mutation rate for an average gene. It is unclear whether wild-type λ was released by cells that were wild type except for the *recA* mutation, since we cannot recover these cells. A few mutants with an elevated but still low switching rate are known (51). Perhaps host mutants exist with a

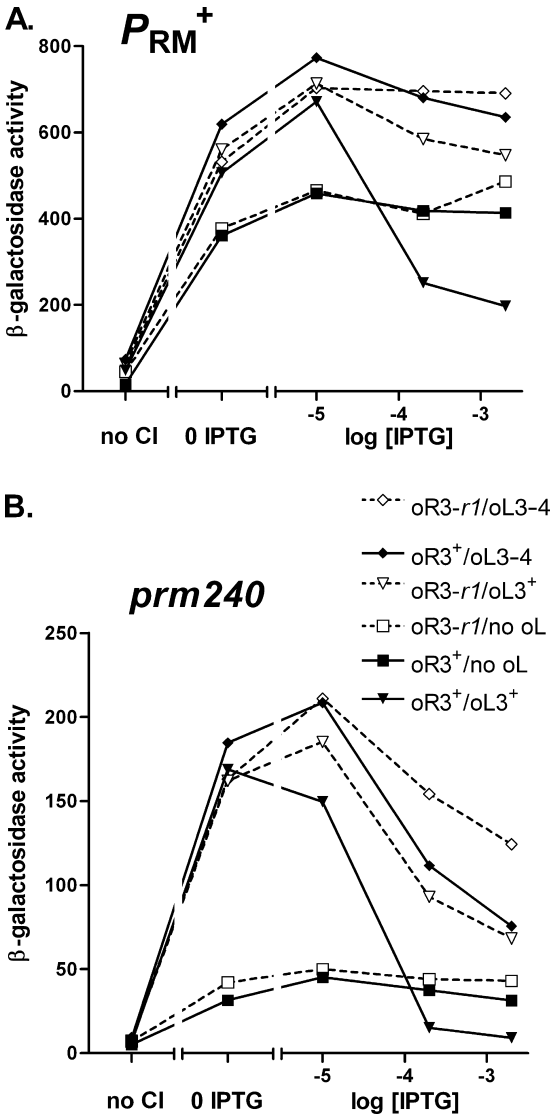

FIG. 4. Positive and negative autoregulation of P_{RM}^+ and *prm240*. Cells were grown at 30°C in M9 glucose; experiments were otherwise as in Fig. 3. Data for P_{RM}^+ (A) and *prm240* (B) were obtained in separate experiments. For each promoter, six reporter strains were used, which differed in the alleles of O_R3 and O_L present, as indicated. Symbols are the same for both panels.

greatly enhanced switching rate but have not been analyzed because they are lethal. We conclude that the lysogenic state is extremely stable and that the rate of switching is so low that a precise measurement would be difficult.

Evolution of stability. Why does λ have such a stable lysogenic state? Evolution almost certainly did not operate on *recA* mutant lysogens. In a *recA*$^+$ host, RecA-dependent spontaneous switching occurs at a much higher rate (see introduction), so there is not an obvious need for the lysogenic state to have such a high intrinsic stability.

We surmise that the stability is tuned to maximize the overall reproductive rate at which phage genomes increase in the environment, whether as free phage or as prophages. The level of SOS activation varies markedly; in addition to the low level of spontaneous switching, the SOS system gives efficient induction after a large dose of DNA damage and an intermediate rate of phage production (the "subinduced" state) in response to low doses of DNA damage, whether applied in a single dose by UV (8) or by chronic treatment with agents such as mitomycin C (see reference 35).

We do not study λ or its host in their natural environments (27, 53), and it is unclear how the level of SOS activation varies in such environments. Since the colon is almost anaerobic (28), there might be less oxidative damage to DNA and less sporadic activation of the SOS system. Conversely, the presence of DNA-damaging agents from the diet (e.g., aflatoxins) or produced by other gut flora might increase the level of SOS activation on occasion. In sum, we do not know how to "integrate" the selective pressures, or the level of SOS induction, operating in the likely range of natural environments, as would be needed to estimate how changes in stability of the circuit would influence the overall rate of phage genome production.

At the mechanistic level, mutations can modulate both the intrinsic stability of the circuitry (see Results) and the rate of CI cleavage (13, 24, 25) (our unpublished data). Different combinations of these two features would likely give essentially the same rate of phage production over the naturally occurring range of SOS-inducing treatments. In particular, a circuit that is less stable might be combined with a lower rate of CI cleavage. Nonetheless, several wild phage isolates with λ immunity specificity have the same regulatory circuitry in the immunity region as λ (15), suggesting that covariation in stability and cleavage rate is not frequent in extant λ immunity phages.

Selection might also operate on the host. Many lambdoid phages carry genes that confer a selective advantage to the host and express these genes, either in the lysogenic state (lysogenic conversion) or after spontaneous induction (38). Lambdoid phages can be considered a single species, with assortment of functional modules on an evolutionary time scale (32). Hence, the immunity region of λ, which is also carried on other phages (15), is likely associated at times with genes that confer a selective advantage on the host. This would select for a highly stable lysogenic state.

Switching threshold. We define the switching threshold as that level of CI at which switching becomes likely. Our data suggest that its value for λ*prm240* is roughly 25% of the level of CI in a wild-type λ lysogen, a value measured at ~250 monomers of CI per cell growing in rich medium (49). That is, the switching threshold for this mutant is in the range of ~60 molecules per cell. This value is rough, because it represents the average of cells in the population, and generally the cells with the lowest values are most likely to switch. It is known (7) that in the wild type the level of CI varies substantially, and it is reasonable to expect at least this much variation for a λ*prm240* lysogen, particularly since negative autoregulation would not act to counteract fluctuations (9, 17).

The value for wild-type λ is almost certainly less than this, since with its stronger P_{RM} it can "fight back" to replenish CI more effectively than λ*prm240* can do. Previous work (8) led to an estimate of 10% of the lysogen level for the switching threshold, based on the amount of CI DNA binding activity found at 30 min after UV, but interpretation of these data involves several assumptions, including knowing the time that cells switch and assuming that all cells switch at the same time.

With this small number of molecules, stochastic events play a large role in determining the fate of the cell (39, 50). That is, there is not a discrete number at which switching invariably occurs; instead, at any given number of CI molecules, there is a certain probability of switching, a probability that increases as the number of CI molecules decreases or the cell size increases. Specific pathways are suggested by the properties of mutants we studied, as we now discuss.

Mechanisms of switching. Though switching of λ*prm240* lysogens likely follows a pathway generally similar to that for prophage induction (see introduction), the low level of CI and the lack of an active mechanism (cleavage) for its removal suggest two additional mechanisms, both involving stochastic effects, that may play a role in some λ*prm240* switching events. First, cell division may reduce abruptly the level of CI in some cells. CI that is free in solution or bound nonspecifically to DNA should partition between progeny cells according to a binomial distribution (50). A cell receiving substantially less than half the CI might lose positive autoregulation, leading soon to switching. This mechanism could not operate during SOS-mediated prophage induction; cell division is blocked during that process by the SulA protein, which is expressed at high levels by the SOS system.

Second, DNA replication may also play a role in switching of λ*prm240*. A cell growing in rich medium usually has four copies of the region where λ integrates (45); hence a lysogen has four prophages, each with six CI binding sites. If four of these sites on each prophage (all but O_R3 and O_L3) are occupied by CI dimers, 32 CI monomers, roughly half the number present at the switching threshold, would be bound specifically as dimers. When these prophages are replicated, two events might contribute to switching by derepressing the lytic promoters. When the replication fork passes the prophage, the number of binding sites doubles. Since initiations at *oriC* are somewhat synchronized (57), replication of the prophage may occur at about the same time on all four chromosomes, and there may not be enough CI to bind fully all the operators. In addition, bound CI will dissociate from the DNA, at least transiently, during passage of the replication fork, possibly leading to some expression of lytic promoters before CI can rebind. Hence, near the switching threshold the mechanics of DNA replication may favor switching, particularly in combination with the potential for unequal CI partitioning upon cell division as discussed above. Again, this mechanism would likely not operate after DNA damage, since the forks are stalled at lesions and would traverse the prophage only in a small fraction of cells. In our view, both the above events (binomial partitioning and effects of DNA replication) should be included in stochastic models of cellular processes.

The effects of secondary mutations on the stability of λ*prm240* help provide insight into the mechanisms of switching. Mutations in O_R1 and O_R2 destabilized λ*prm240*, an effect likely resulting from derepression of the lytic promoters at somewhat higher levels of CI; that is, the switching threshold was probably higher. In contrast, mutations eliminating negative autoregulation stabilized λ*prm240*. They presumably led to somewhat higher CI levels, making expression of lytic promoters somewhat less likely.

More puzzling is the finding that *cro-z8*, a mutation leading to lower levels of Cro protein, reduced only modestly the switching rate of λ*prm240*. Two lines of evidence suggested that this mutation would have a large stabilizing effect. First, it was isolated (see Fig. S2 in the supplemental material) from a mutant phage that could not form stable lysogens, acting as a suppressor that allowed lysogenization. Second, λ*c*I Y210N, with a mutation that eliminates cooperative CI binding, cannot lysogenize (6); we found here that the *c*I Y210N *cro-z8* double mutant formed turbid plaques and displayed the unstable lysogen phenotype, so that *cro-z8* suppressed the lysogenization defect. Hence, in both cases, *cro-z8* shifts the balance toward the lysogenic state.

We suggest several possibilities for the modest effect of *cro-z8* on the stability of λ*prm240*. Perhaps the *cro-z8* mutation provides adequate levels of Cro to give some repression of P_{RM}. Alternatively, Cro-mediated repression of P_{RM} might not be important for switching of λ*prm240*, in apparent contrast to the effects of *cro* mutations in the two contexts just mentioned. One likely difference is that these phages carried P_{RM}^+, while cells containing *prm240* should have far lower levels of CI. Some switching events might occur as a result of stochastic processes based on cell division or DNA replication, and Cro-mediated repression of P_{RM} might not be important in these cases. The finding (Table 5) that *cro-z8* has a smaller effect at higher rates of switching is consistent with this suggestion. A final possibility is that, despite its low average expression rate, *cro-z8* may allow wild-type levels of Cro in a small fraction of cells, making enough Cro to support switching in those cells. Perhaps the promoter is expressed in bursts, as observed for other promoters (26), or the secondary structure of the mutant mRNA may allow it to have switch-like behavior, alternating between translationally active and inactive forms.

Properties of the *prm240* promoter. We found that *prm240* was regulated in response to CI similarly to P_{RM}^+ (Fig. 3 and 4), with two quantitative differences. First, the degree of stimulation by looping was greater. This finding suggests that, whatever the mechanism by which looping stimulates P_{RM}, it is substantially more effective with *prm240*, and further analysis of *prm240* may help determine this mechanism. Second, *prm240* was more sensitive to negative autoregulation than WT P_{RM} (Fig. 4). This pattern suggests that CI can bind more effectively after initiation of transcription on the mutant promoter than on its wild-type counterpart. A plausible mechanism is that CI can compete more successfully with RNA polymerase for binding to the weaker promoter; it is known that the primary effect of *prm116*, another P_{RM} down-mutation affecting this same position in the −35 region, is to weaken RNA polymerase binding (55).

Substantial repression of *prm240* was observed (Fig. 4) in the presence of the O_R3-*r1* mutation, which weakens CI binding to O_R3 ~100-fold (17, 52). We expect a less severe effect on CI binding to the mutated site in the context of a dodecamer, since multiple weak interactions in the complex will partially compensate (21). Even with the O_R3-*r1* O_L3 template, with no specific binding to O_L3, some repression remained, suggesting that a repressive complex could form in the absence of specific CI-O_L3 interactions; again, multiple cooperative interactions among the components, and residual nonspecific binding of CI to O_L3 or a nearby sequence, might make this possible. Hence, the finding that no repression is observed on this template with WT P_{RM} implies that RNA polymerase is bound nearly all the

time to WT P_{RM}; otherwise, the same repressive complex could form and confer some repression.

Properties of λ*prm240*. The stability of λ*prm240* lysogens was markedly responsive to temperature and growth rate. Several findings suggest that these responses reflect a more general aspect of cell and/or viral physiology. First, other P_{RM} mutations conferred similar behavior (see Results). Second, another mutant, λJL188 (see Table 1), also exhibited the unstable lysogen phenotype. It has four mutations in the O_R1 site, which should affect the binding of CI and Cro and the strength of the P_R promoter. Hence, these conditional responses can arise from changes in other *cis*-acting sites. Third, free phage levels made by a *recA* mutant (λO_R323) lysogen were ~200-fold lower when cells were grown in minimal medium (unpublished data), though the actual difference is likely less than this, due to instability of λ virions in this medium (see Results). Finally, preliminary data suggest that the set point (see reference 42) for UV induction of a *recA*$^+$ (λO_R323) lysogen is markedly higher in minimal medium than in LB medium. In sum, the responses of λ*prm240* to growth conditions are not specific to the mutation in that phage. We speculate that the λ circuitry has evolved to fine-tune the probability of induction in response to the growth conditions.

Why does *prm240* appear so frequently? We found it in every culture examined. It is likely a hot spot for mutation. In addition, λ*prm240* lysogens, once arisen, have another property that should contribute to the abundance of free mutant phage. If we consider the relationship between the switching rate of various mutants and the resulting titer of phage, intuitively a mutant with an intermediate switching rate should yield the highest titer of phage. If the rate is zero (as is nearly so with WT), no free phage should result. If a mutant always switches as a result of a newly arising *c*I mutation, the cell would give no progeny, and one might obtain ~5 to 10 bursts in the lysate, yielding ~500 to 1,000 phage. For lysogens with switching rates between these extreme values, some mutant cells would switch and some would divide to give two daughter cells. Hence, the number of cells capable of releasing free phage would continue to increase, as would the number of free phage. This increase should occur in the presence of a large excess of JL5904, just as is observed with the λ*prm240* lysogen by itself (Fig. 2). Hence, there should be an optimal switching rate giving the highest titer of free phage.

We verified this expectation by simulating the relationship between switching rate and total phage titer, as described in the supplemental material. This simulation also showed that the optimum switching rate depended on the length of time the culture was grown, becoming progressively lower with longer growth times (see Fig. S4 in the supplemental material). Hence, it is plausible that a mutant could exist with an optimum switching rate. Though it is unclear whether *prm240* is such a mutant, the use of λ*prm240* derivatives with altered degrees of stability should enable tests of this expectation.

ACKNOWLEDGMENTS

We are grateful to Meadow Anderson, Adam Arkin, John Clark, Carol Dieckmann, Telsa Mittelmeier, and Bruce Patterson for helpful discussions; to Andrew Capaldi, Carol Dieckmann, Don Court, Ian Dodd, Gary Gussin, Telsa Mittelmeier, and Lynn Thomason for comments on the manuscript; and to Meadow Anderson and Haw Yang for communicating results before publication.

This work was supported by grant GM24178 from the National Institutes of Health.

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