Octamerization of λ CI repressor is needed for effective repression of P_{RM} and efficient switching from lysogeny

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The CI repressor of bacteriophage λ is a model for the role of cooperativity in the efficient functioning of genetic switches. Pairs of CI dimers interact to cooperatively occupy adjacent operator sites at O_R and at O_L . These CI tetramers repress the lytic promoters and activate transcription of the *cI* gene from P_{RM} . CI is also able to octamerize, forming a large DNA loop between O_R and O_L , but the physiological role of this is unclear. Another puzzle is that, although a dimer of CI is able to repress P_{RM} by binding to the third operator at O_R , O_{R3} , this binding seems too weak to affect CI production in the lysogenic state. Here we show that repression of P_{RM} at lysogenic CI concentrations is absolutely dependent on O_L , in this case 3.8 kb away. A mutant defective in this CI negative autoregulation forms a lysogen with elevated CI levels that cannot efficiently switch from lysogeny to lytic development. Our results invalidate previous evidence that Cro binding to O_{R3} is important in prophage induction. We propose the octameric CI: O_R - O_L complex increases the affinity of CI for O_{R3} by allowing a CI tetramer to link O_{R3} and the third operator at O_L , O_{L3} .

[Key Words: CI repressor; octamer; DNA looping; genetic switch; negative autoregulation; bacteriophage λ]

Received August 14, 2001; revised version accepted September 28, 2001.

Bacteriophage lambda occupies a special place in molecular biology, having been an immensely productive model system in our understanding of many fundamental biological processes (for recent review, see Friedman and Court 2001). Of particular value has been the unraveling of the gene regulatory mechanisms and strategies that comprise lambda's genetic switch, a bistable switch that underlies the phage's ability to efficiently use its two modes of development (Ptashne 1992). In the emerging field of gene network analysis, the lambda regulatory network has often been used as a testing ground for modeling methods (for review, see Hasty et al. 2001). However, despite the extensive characterization of the lambda switch, a few of its features remain unexplained. In the present study we show that some of these features combine in a regulatory mechanism necessary for efficient switching from lysogeny to lytic development.

The key regulator in the λ genetic switch is the CI protein, which binds to the O_R and O_L regions of the λ genome to repress the lytic promoters during lysogeny. Cooperative binding of a pair of CI dimers at two of the three CI operators at O_R (O_{R1} and O_{R2}) represses P_R (Fig. 1; Johnson et al. 1981; Ptashne 1992), and similar binding at two of the three operators at O_L (O_{L1} and O_{L2}) re-

presses the P_L promoter (Johnson 1980; Brenowitz et al. 1986). Binding of CI to O_{R2} also stimulates the intrinsically weak promoter for the *cI* gene, P_{RM} (Meyer and Ptashne 1980), so that in the lysogenic state CI positively regulates its own synthesis and the presence of CI is maintained. Switching from lysogeny to lytic development, called lysogenic induction or prophage induction, occurs on activation of the host SOS system in response to DNA damage, for example, after treatment with ultraviolet light (UV). RecA bound to ssDNA stimulates the self-cleavage of CI monomers (Roberts and Devoret 1983; Little 1984), removing CI's ability to bind DNA cooperatively and reducing the activity of CI to a point where the lytic promoters become derepressed.

CI is also capable of negative autoregulation when at high concentrations, repressing the transcription of its own gene from P_{RM} by occupying the third operator at O_R , O_{R3} (Fig. 1; Maurer et al. 1980). However, despite the existence of this regulatory mechanism, the studies of Maurer et al. (1980) revealed very little negative autoregulation of CI—at the lysogenic CI concentration, the activity of P_{RM} was reduced only ~5%–20% from maximal, and repression of P_{RM} to half its maximal activity required a CI concentration 15 times the normal lysogenic level. It has therefore become accepted that CI negative autoregulation has little or no physiological role in the lysogenic state (Johnson et al. 1981; Ptashne 1992; Darling et al. 2000). Thus, it may be that O_{R3} functions primarily as a site for repression of P_{RM} by Cro

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.937301>.

Figure 1. Regulation of *ci* transcription in the lysogenic state. The upper cartoon shows a CI octamer occupying the O_L1 , O_L2 , O_R1 , and O_R2 operators. Transcription of the *ci* gene from the P_{RM} promoter by RNA polymerase (RNAP) is stimulated by CI bound at O_R2 but repressed by CI bound at O_R3 . The lower portion shows the sequence of P_{RM} and O_R3 , and the mutations used in this study with the predicted changes in free energy of binding of CI and Cro.

protein (Johnson et al. 1981; Ptashne 1992), with CI binding to O_R3 being a by-product of CI and Cro's ability to bind to similar DNA sequences.

The function of the third operator at O_L , O_L3 , is also unclear. The contribution of O_L3 to CI repression of P_L is probably small (Brenowitz et al. 1986), and Cro binding to O_L3 is unlikely to affect P_L , so that two CI operators should suffice at O_L . It has been suggested that having three CI operators at O_L and O_R provides a redundancy in CI repression of the lytic promoters that makes it more difficult for virulent strains of λ to arise by mutation (Johnson et al. 1981).

Recently, Révet et al. (1999) demonstrated that CI is able to form a long DNA loop between O_L and O_R . They showed by electron microscopy that a DNA loop can form in vitro between two pairs of CI dimers spaced 2.5 or 2.9 kb apart (the natural O_L and O_R spacing is 2.4 kb). Using reporter studies, they showed that the presence of O_L1 and O_L2 at a distance of 3.6 kb from P_R enhanced CI repression of P_R in vivo fourfold. The requirement for four CI operators led Révet et al. (1999) to propose that the DNA looping in their experiments was mediated by a CI octamer, as shown in Figure 1. This is consistent with the association properties of CI in solution (Senear et al. 1993) and with the crystal structure of the CI C-terminal domain (Bell et al. 2000). However, the physiological relevance of CI octamerization is not clear (Senear et al. 1993; Bell et al. 2000; Koudelka 2000) because P_R is 98.5% repressed by lysogenic CI concentrations even in the absence of O_L (Meyer et al. 1980).

Here we show by reporter studies that CI at its lyso-

genic concentration does repress P_{RM} , but only in the presence of O_L , placed, in this case, 3.8 kb away. A phage with a mutation in O_R3 that eliminates the ability of CI to repress P_{RM} forms a lysogen that produces elevated CI levels and is defective in prophage induction by UV. We propose that the formation of the O_L - O_R loop through CI octamerization juxtaposes O_R3 and O_L3 such that they can be occupied cooperatively by a CI tetramer, allowing effective repression of P_{RM} . This mechanism acts to limit the CI concentration in the lysogenic state and permit efficient switching into lytic development.

Results

Mutations in O_R3 affect the efficiency of prophage induction

We reasoned that if CI negative autoregulation is not significant in lysogeny, as widely believed, then mutations in O_R3 that alter the ability of CI to repress P_{RM} should have little or no effect on the properties of the lysogenic state. Mutations designed to increase or decrease CI repression of P_{RM} were therefore introduced into O_R3 (Fig. 1). The analyses by Takeda and coworkers of the effects of base pair changes in O_R1 on the strengths of CI and Cro binding (Sarai and Takeda 1989; Takeda et al. 1989) were used as a guide, with the reservation that the effects may be different in the context of O_R3 . We chose *r1* as a mutation that should markedly decrease CI binding with little effect on Cro binding. The *r1* mutation has been previously shown to strongly reduce repression of P_{RM} by CI (Maurer et al. 1980). The *c12* change should improve CI binding slightly while decreasing Cro binding. The mutations were created in a plasmid vector and then the wild-type and mutant operator regions were recombined in vivo into λ^+ to give λ_{wt} , λ_{r1} , and λ_{c12} .

The three phages formed indistinguishable turbid plaques on indicator bacteria. Phage production kinetics after infection of the bacterial strain C600 were unaffected by the mutations (Fig. 2A). Monolysogens of C600 and NK7049 were obtained without difficulty and the UV-inducibility of the wild-type and mutant prophages in these lysogens was compared. Phage production after treatment of the C600 lysogens with 7.6 J/m² UV was much less efficient in the λ_{r1} lysogen, whereas the λ_{c12} mutant was induced slightly better (Fig. 2B). Compared with the λ_{wt} lysogens, fewer λ_{r1} lysogens were induced (29% of wild type: 95% confidence limits 14%–52%, $n = 4$), the average number of phage produced per induced lysogen was much less (9% of wild type: 95% confidence limits 2%–24%), and the time required to release phage particles was longer (~60 min compared with 45 min). The fraction of NK7049 λ_{r1} lysogens induced after UV doses of 5.6–11.2 J/m² was ~10% of λ_{wt} , whereas the λ_{c12} mutant was induced better than λ_{wt} at all doses (Fig. 2C), the maximal difference being 1.7-fold at 5.6 J/m² ($P < 0.05$, 2-tailed t -test, $n = 4$). Thus the *r1* and *c12* mutations at O_R3 have significant and opposite effects on the ability of λ to exit the lysogenic state in response to UV light.

Figure 2. Defective ultraviolet (UV) induction of O_R3 mutant prophages. (A) Infection of C600. Plaque-forming units (PFU) per milliliter after infection of C600 with λ wt, λ r1, or λ c12 (averages of two experiments). (B) UV-induced phage production from C600 lysogens. PFU per milliliter produced after UV irradiation (7.6 J/m²) of C600 lysogenic for λ wt, λ r1, or λ c12 (averages of four experiments). (C) UV-induced phage production from NK7049 lysogens. Fraction of lysogens producing phage versus UV dose (averages of four experiments).

Mutations in O_R3 affect the CI concentration in the lysogenic state

The effects of the O_R3 mutations on prophage induction could be explained if the concentration of CI in the lysogenic state was increased by the $r1$ mutation and decreased by the $c12$ mutation, because this would hinder or assist, respectively, clearance of CI activity from the cell by activated RecA. To test this, we directly measured CI levels in the λ wt, λ r1, and λ c12 NK7049 lysogens by two methods: a gel mobility shift assay for DNA binding activity and Western blotting (Fig. 3). In both methods, extracts from λ wt lysogenic cells and

nonlysogenic cells were mixed to produce a set of standards, and extracts from the λ r1 and λ c12 lysogens were diluted in nonlysogenic extract and compared with these standards. This allowed determination of CI levels or activities in wild-type lysogenic units (WLU), that is, relative to the wild-type lysogen. The two methods gave good agreement. The gel shift assays gave CI levels in the λ r1 and λ c12 lysogens as 2.8 and 0.6 CI WLU, respectively (95% confidence limits: $r1$ 2.43–3.14 and $c12$ 0.51–0.67, $n = 4$). Immunoblotting gave CI levels in the λ r1 and λ c12 lysogens as 2.3 and 0.6 CI WLU, respectively (95% confidence limits: $r1$ 2.10–2.53, $n = 5$, and $c12$ 0.45–0.74, $n = 6$). Thus, the O_R3 mutations have strong effects on the lysogenic CI concentration.

Figure 3. Effect of O_R3 mutations on lysogenic CI levels. (A) Gel shift quantitation of CI concentrations in λ lysogens. Extracts from NK7049 λ wt, λ r1, and λ c12 lysogens (with plasmids pUHA1 and pZC320) were diluted in nonlysogenic extract; the dilution factor indicates the proportion of lysogenic extract in each reaction. The fraction of the 154-bp O_L -containing DNA fragment bound in each lane was measured, allowing determination of the CI levels in the $r1$ and $c12$ extracts relative to wt, that is, in CI wild-type lysogenic units (CI WLU, see Materials and Methods). (B) Western blotting to quantitate CI concentrations in λ lysogens. Extracts were as in (A) except that pZC320 was sometimes replaced by pZE15. The two leftmost tracks are from a separate blot and only a portion of the blots is shown.

CI transcriptional autoregulation in the absence of O_L

The effects of the $r1$ and $c12$ mutations on the concentration of CI in the lysogenic state are consistent with their expected effects on CI repression of P_{RM} . The $c12$ mutation should increase CI repression of P_{RM} , leading to a lower CI expression in the lysogen; the $r1$ mutation should decrease CI repression of P_{RM} and increase CI levels. However, the magnitude of the $r1$ effect, an ~250% increase in CI levels, was inconsistent with the finding of Maurer et al. (1980) that no more than 20% repression of P_{RM} occurred in the lysogenic state. To try to resolve this discrepancy, we repeated the experiment of Maurer et al. (1980) by examining the activity of the wild-type and mutant P_{RM} promoters in vivo in response to a range of CI concentrations.

To report on the activity of P_{RM} , we cloned DNA fragments from the –123 to the +62 positions of the promoter (+42 to –143 of P_R) upstream of *lacZ* and placed them in single copy in the bacterial chromosome of NK7049 as part of a λ prophage (Fig. 4A), using a modification of the system of Simons et al. (1987). Note that the reporter integrates at the λatt site but, since it carries the immunity region of $\phi 21$ (*imm21*), it does not make λ CI and does not contain λO_R or O_L . To test the effect of a range of CI concentrations on P_{RM} , we supplied CI to these reporters from either of two plasmids carrying the *cI* gene under the control of the wild-type *Plac* promoter. Plasmid pZC320*cI* is a single copy, mini-F based plasmid (Shi and Biek 1995), and pZE15*cI* has a copy number of

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Figure 4. The effect of O_{R3} mutations on CI regulation of P_{RM} in the absence and presence of O_L . (A) The $P_{RM}::lacZ$ reporter system. The $P_{RM}::lacZ$ operon fusion was present on a single copy λimm^{21} prophage in NK7049. The $lacY$ and $lacA$ genes were removed by deletion. O_L , when present, was inserted at the deletion point and was 3.8 kb downstream of O_R . All strains in (B) to (D) were NK7049, carrying a reporter prophage and plasmid pUHA1 (makes Lac repressor) and the P_{lac} CI expression plasmid pZC320cI or its cI -less parent, pZC320. (B) CI levels produced by the pZC320cI plasmid. Extracts of (λ RS45 Δ YA- $P_{RM}wt::lacZ$) with pZC320cI, grown with the indicated IPTG concentrations, were analyzed for CI concentration relative to the wild-type lysogenic level by the gel shift assay. Each point represents the mean of at least two determinations of CI levels from one extract. The curve is the best fit to a rectangular hyperbola (Sigmaplot). (WLU) Wild-type lysogenic units. (C) Activity of wt and mutant P_{RM} promoters in the absence of O_L . LacZ activities of (λ RS45 Δ YA- $P_{RM}::lacZ$) pZC320cI versus CI concentration produced by IPTG induction according to the relationship of (B). Points are the means of three experiments. The zero CI points are the basal activities of the wt and mutant P_{RM} promoters (in the presence of pZC320 and no IPTG). (D) As (C) but in the presence of O_L , that is, with (λ RS45 Δ YA- O_L - $P_{RM}::lacZ$). Points are the means of 4–10 experiments; the error bars show the 95% confidence limits. The solid symbols to the right of the curves are the mean LacZ activities of the P_{RM} reporters in the presence of lysogenic CI levels produced by a $\lambda att80$ prophage (with pZC320 and IPTG concentrations ranging from 50 to 500 μ M).

50–70 (Lutz and Bujard 1997). In each strain, a second plasmid, pUHA1, carried the wild-type lac repressor gene, allowing IPTG control of CI expression. Western blots (data not shown) and gel shift assays (summarized in Fig. 4B) showed that pZC320cI gave good control of CI expression, ranging from about 0.06 CI wild-type lysogenic units in the absence of IPTG to ~ 3 CI WLU at 300 μ M IPTG, with the lysogenic CI concentration produced at 60 μ M IPTG. In contrast, Western blots (data not shown) indicated that plasmid pZE15cI could be induced to produce at least 20 CI WLU.

Figure 4C shows the activity of the wt, $r1$, and $c12$ P_{RM} promoters in the presence of increasing IPTG induction of CI expression from pZC320cI. The IPTG concentrations have been converted to CI concentrations using the empirical relationship of Figure 4B. The mutations did not strongly affect the basal activity of P_{RM} . Over the range of CI concentrations produced by pZC320cI, the wild-type and mutant P_{RM} promoters were activated but not repressed. All the promoters reached maximal activity at about the lysogenic CI concentration, but increasing CI levels produced no reduction in activity, even in the $c12$ mutant (Fig. 4C). These data support the observation of Maurer et al. (1980) that very little or no re-

pression of P_{RM} occurs at the lysogenic CI level. In accord with Maurer et al. (1980), we did see repression of wt and $c12$ P_{RM} when we supplied very high levels of CI with pZE15cI (data not shown). The $c12$ mutation improved the efficiency of repression of P_{RM} , consistent with its expected effect on CI binding at O_{R3} . However, the $r1$ mutant P_{RM} remained at maximal activity even at the very high CI concentrations, confirming that the $r1$ mutation eliminates repression of P_{RM} .

Judging from the maximal P_{RM} activities under conditions in which there was no apparent occupation of O_{R3} (Fig. 4C), the mutations appear also to affect the ability of P_{RM} to be activated by CI. The $r1$ mutation improved maximal P_{RM} activity by $\sim 9\%$, whereas the $c12$ mutation reduced it by $\sim 30\%$. Thus, the $r1$ mutation, at the -17 position of P_{RM} , seems to slightly improve the ability of RNA polymerase at P_{RM} to be activated by CI bound at O_{R2} , whereas the $c12$ mutation, which alters the -25 position of P_{RM} , may interfere with the response of RNA polymerase to CI at O_{R2} .

The regulation of P_{RM} by CI seen in our $lacZ$ reporter assays in the absence of O_L confirms the discrepancy with our measurements of lysogenic CI levels. The $\lambda r1$ prophage produced a CI level that was 2.3–2.8-fold that

of the λ wt prophage, yet the activity of P_{RM-r1} at 2.3–2.8 CI WLU was no more than 1.1 times that of P_{RM-wt} at 1 CI WLU. This small increase in P_{RM} activity due to the $r1$ mutation is unable to explain the large increase in CI concentration in the $\lambda r1$ lysogen.

CI transcriptional autoregulation in the presence of O_L

We reasoned that if the CI-mediated DNA loop between O_R and O_L (Révet et al. 1999) occurs in the prophage DNA, then O_L may affect CI regulation of P_{RM} in the lysogenic state. We therefore introduced O_L downstream of *lacZ* in our reporter constructs (Fig. 4A). The O_L operators were placed in the same orientation relative to the O_R operators as they are in the phage, but O_L and O_R were spaced 3.8 kb apart rather than the natural 2.8-kb distance. Like Révet et al. (1999), we mutationally inactivated the P_L promoter on the O_L fragment, but we included all three O_L operators, rather than just O_{L1} and O_{L2} .

The P_{RM} activities versus CI concentration in the presence of O_L are shown in Figure 4D. Repression of the wild-type and *c12* P_{RM} promoters was much more efficient in the presence of O_L (Fig. 4, cf. C and D). At the lysogenic CI concentration (1 CI WLU), wild-type P_{RM} activity was reduced 16% from its maximal level and the activity of *c12* P_{RM} was reduced 36% from its maximal level. However, even in the presence of O_L , P_{RM} in the *r1* mutant remained resistant to repression. This shows that the improvement of repression caused by O_L requires an intact O_{R3} , indicating that O_L is acting by increasing CI occupation of O_{R3} . The magnitude of the effect of O_{R3} on P_{RM} repression can be seen by comparing *wt* and *r1* P_{RM} . At the lysogenic CI concentration, the activity of wild-type P_{RM-wt} was 34% lower than the activity seen in the *r1* mutant. Thus, assuming that O_{R3} is completely unoccupied in the *r1* mutant, and allowing for the 9% improvement of P_{RM} activation by CI caused by the *r1* mutation, we can estimate that O_{R3} is occupied 26% of the time in the lysogen.

The negative autoregulation by CI in the presence of O_L goes some way to explaining the difference in CI concentration between the λ wt and $\lambda r1$ lysogens. From the data of Figure 4D, the expected activity of P_{RM-r1} in the $\lambda r1$ lysogen (at 2.3–2.8 CI WLU) is ~1.5 times the expected activity of P_{RM-wt} in the λ wt lysogen (at 1 CI WLU). Assuming that CI production is proportional to P_{RM} activity quantitated by LacZ units, we would therefore predict the concentration of CI in the $\lambda r1$ lysogen to be 1.5 times that of the λ wt lysogen, a value ~60% of the measured CI level. A similar argument predicts the CI concentration in the $\lambda c12$ lysogen to be 0.6 that of wild type, matching the measurement of CI levels for the $\lambda c12$ lysogen (0.6 CI WLU).

As a check on our determination that the wild-type lysogenic CI level is produced from pZC320*cI* at 60 μ M IPTG (Fig. 4B), we supplied the lysogenic CI level to the reporter constructs from a single-copy λ prophage ($\lambda att80$) integrated at the $\phi 80$ chromosomal attachment site. The activities of the *wt* and mutant P_{RM} reporters in

the presence of this prophage are shown to the right of the curves in Figure 4D. Surprisingly, the activities of the P_{RM-wt} and P_{RM-c12} promoters were considerably lower than those obtained with 1 CI WLU from pZC320*cI*. In fact, all three promoters showed activities similar to those seen at 2 CI WLU with pZC320*cI*. Yet the activity of CI in the $\lambda att80$ lysogen, as measured by the gel shift assay, was similar to that produced from pZC320*cI* at 60 μ M IPTG (measurements of two extracts gave values of 1.0 and 1.1 CI WLU). Maurer et al. (1980) reported a similar anomaly between CI activity in *lacZ* reporter assays, depending on whether CI was produced from a plasmid or a prophage. We believe that the anomaly is due to the plasmid-produced CI level being prone to cell-to-cell variability. Large cell-to-cell variation can occur in levels of proteins produced from expression plasmids and this variation has the effect of smoothing activity versus concentration curves, such as those in Figure 4, C and D (Cluzel et al. 2000). Thus, the culture with pZC320*cI* at 60 μ M IPTG has the same average CI content as a lysogen, but the population may be a mix of cells: those with less CI and a higher-than-lysogenic P_{RM} activity and those with more CI and similar-to-lysogenic P_{RM} activity. The average P_{RM} activity of such a population would overestimate the true activity at the lysogenic concentration. In contrast, we expect the native λcI gene on the $\lambda att80$ prophage to produce a more consistent CI level. This is because negative autoregulation by a protein has the effect of reducing fluctuation around its steady state concentration (see Discussion), as demonstrated experimentally by Becskei and Serrano (2000). We therefore expect that the P_{RM} activities in the presence of the $\lambda att80$ prophage more truly reflect the lysogenic state.

In support of this, a lower activity of *wt* P_{RM} in the lysogenic state resolves the discrepancy between the reporter data and the lysogenic CI concentrations. The expected activity of P_{RM-r1} in the $\lambda r1$ lysogen becomes ~2.4 times the expected activity of P_{RM-wt} in the λ wt lysogen, which matches the observed 2.3–2.8-fold difference in CI concentration. The predicted CI concentration in the $\lambda c12$ lysogen becomes ~0.5 times that of wild type and thus remains consistent with measurement.

On this basis, CI negative autoregulation in the lysogenic state is more severe: in the presence of the $\lambda att80$ prophage, P_{RM-wt} activity was reduced 58% from the activity of P_{RM-r1} (Fig. 4D), giving an estimate of O_{R3} occupation in the wild-type lysogen of 53% (see above).

The effect of the O_{R3} mutants on Cro repression of P_{RM}

The effects of the O_{R3} mutations on the efficiency of prophage induction can be explained by their effects on the CI concentration in the lysogenic state. However, the mutations may alter prophage induction for a different reason: by changing the ability of P_{RM} to be repressed by the early lytic protein Cro. Cro binds more tightly to O_{R3} than to the other λ operators (Takeda et al. 1989; Darling et al. 2000), and it has been suggested that Cro

produced early in prophage induction binds to O_R3 to repress P_{RM} , preventing further CI synthesis and thus aiding CI removal (Johnson et al. 1981; Ptashne 1992). By this reasoning, the *c12* mutation, which should reduce Cro binding, should cause an impairment of prophage induction rather than the improvement that we saw. Nevertheless, we wished to check whether differences in Cro repression of P_{RM} might have impact on the differences in prophage induction found between the wild-type and two mutant phages. The *cro* gene was therefore cloned into the high-copy pZE15 expression vector and the effect of Cro on basal P_{RM} transcription in the presence of O_L was examined (Fig. 5). Wild-type P_{RM} was effectively repressed by Cro. The *r1* mutation had no significant effect, whereas the *c12* mutation caused a slight reduction in the efficiency of Cro repression of P_{RM} . It is clear, then, that the effects of the two mutations on prophage induction cannot be explained by differences in Cro repression of P_{RM} . Friedman and Court (2001) have suggested that Cro repression of P_{RM} might be affected by O_L , but no significant changes were seen on repeating the experiment in the absence of O_L (data not shown).

Discussion

We have shown that the efficiency of CI repression of P_{RM} is increased by O_L to the point that P_{RM} is ~50% repressed at the lysogenic CI concentration. The *r1* mutation in O_R3 abolishes this negative autoregulation, resulting in a 2.3–2.8-fold increase in the CI level in the lysogen and causing a strong defect in prophage induction. These findings have important implications for the role and structure of the O_R –CI– O_L DNA loop and for the function of O_R3 and O_L3 , and illustrate the utility of negative autoregulation in allowing efficient switching between developmental states.

Figure 5. The effect of O_R3 mutations on Cro repression of P_{RM} . Activity of wt or mutant P_{RM} promoters with IPTG induction of Cro expression in NK7049(λ RS45 Δ YAO $_L$ - P_{RM} ::*lacZ*) with pUHA1 and pZE15*cro*. Points are the means of four or five experiments; the 95% confidence limits for the last three highest [IPTG] are shown.

The CI-mediated O_L – O_R DNA loop

As pointed out by Bell et al. (2000), the experiments of Révet et al. (1999) do not show that the CI-mediated O_L – O_R loop is important in phage development. Our results show that the O_L – O_R interaction, presumably via the DNA loop, plays a physiologically relevant role in the phage. The large increase in lysogenic CI concentration caused by the *r1* mutation can only be rationalized if there is substantial repression of P_{RM} by CI in the wild-type prophage, and our reporter studies show that such repression requires O_L . In the absence of O_L , we saw no repression of P_{RM} by CI at concentrations close to the lysogenic level, whereas, in the presence of O_L , CI supplied from the λ att80 prophage reduced P_{RM} activity 47% from its maximal observed value. The *r1* mutation abolished this repression, showing that the O_L effect is mediated through O_R3 . Comparison with the activity of P_{RM} -*r1* indicated that O_R3 is 53% occupied by CI in the wild-type lysogen.

Révet et al. (1999) showed that four operators were needed for the O_L – O_R interaction in vivo, suggesting that the loop was mediated by a CI octamer. CI is known to associate to octamers in solution (Senear et al. 1993), and the recently described structure of a tetramer of the CI C-terminal domain revealed surfaces that could provide for such a tetramer:tetramer interaction (Bell et al. 2000). The simplest model to explain our results is that a CI octamer linking its preferred binding sites, O_L1 , O_L2 , O_R1 , and O_R2 (see Fig. 1), may juxtapose O_L3 and O_R3 such that they can be linked by a CI tetramer. Such an interaction between CI dimers bound to O_L3 and O_R3 is consistent with the ability of CI dimers to interact across a short DNA loop (Hochschild and Ptashne 1986). In this model, O_L3 would play a central role in CI negative autoregulation, and we predict that phage defective in CI binding at O_L3 will behave like the λ r1 mutant. Experiments to test this prediction are in progress.

The O_L – O_R interaction is a striking example of gene regulation by a distal site. As far as we are aware, a DNA loop of 2.4 kb is the longest natural, repressive DNA loop known in prokaryotes. We expect that a stable loop of this size requires the large free energy change due to CI octamerization (Senear et al. 1993). Also, repression is completely dependent on the distal site—we saw no repression of P_{RM} at physiological CI concentrations in the absence of O_L . In the absence of cooperative interactions supplied by O_L , the affinity of CI for O_R3 is presumably too low for it to compete with RNA polymerase.

Our results support the idea that the fourfold enhancement of CI repression of P_R by O_L seen by Révet et al. (1999) and, presumably, a reciprocal enhancement of P_L repression by O_R , will occur in the phage context. The linking of O_L3 and O_R3 by a CI tetramer should help to stabilize the O_L – O_R loop and give an even greater improvement of repression of the lytic promoters. We suspect that the effect of the O_L – O_R loop on repression of the lytic promoters is physiologically significant. To stably maintain the lysogenic state, CI must repress P_R tightly to prevent production of Cro; otherwise Cro

would bind to O_{R3} and repress P_{RM} , turning off CI production. Reinitz and Vaisnys (1990) measured the efficiency of Cro production from P_R in the absence of CI and calculated that the activity of CI in the lysogenic state, on the basis of the data of Maurer et al. (1980), would be too low to repress Cro production sufficiently. Our data and that of Révet et al. (1999) show that O_L increases the activity of CI at O_R , helping to explain how CI can maintain effective repression of P_R . Ptashne (1992) has argued that CI cooperativity is critical for switching because it provides for large changes in P_R activity (and P_{RM} activity) in response to relatively small changes in CI concentration. The extra level of cooperativity introduced by CI octamerization may add significantly to this effect.

It is clear that O_L has a strong effect on CI repression of P_{RM} . Does O_L also affect CI activation of P_{RM} ? Friedman and Court (2001) suggested that activation of P_{RM} by CI at O_{R2} might be improved by octamerization of CI through interaction with O_L . We found the opposite effect. The $r1$ mutation eliminates repression of P_{RM} , and therefore reveals an effect of O_L on activation of P_{RM} . We found a drop of ~28% in maximal P_{RM} - $r1$ activity when O_L was introduced (Fig. 4C and D), indicating that O_L reduces activation of P_{RM} . This effect is similar to, but much weaker than, the disruption to P_{RM} activation seen previously when CI at O_{R2} interacts with a CI dimer located 6 or 7 DNA turns further upstream (Hochschild and Ptashne 1988).

The role of O_{R3} in switching from lysogeny to lytic development

Johnson et al. (1981) and Ptashne (1992) postulated that the binding of Cro to O_{R3} is important in efficient switching from the lysogenic to the lytic state. The idea is that Cro produced during the early stages of prophage induction could bind to O_{R3} and repress P_{RM} , preventing further synthesis of CI and thus aiding its removal. This proposal was based on experiments described by Johnson (1980) that used a λ phage carrying two mutations in O_{R3} : the $c12$ mutation and the $r1$ mutation. It was shown that this λO_{R3} - $c12r1$ mutant was strongly defective in UV induction of the prophage. Because it was believed from the results of Maurer et al. (1980) that any effect of the $r1$ mutation on CI repression of P_{RM} was not significant in the lysogen, the prophage induction defect was attributed to the $c12$ mutation and a lack of Cro repression of P_{RM} . However, our results show that the $r1$ mutation strongly impairs prophage induction without affecting Cro repression of P_{RM} , whereas the $c12$ mutation, which slightly impairs Cro repression of P_{RM} , gives an improvement in prophage induction. Experiments with our own $\lambda r1c12$ double mutant indicate a similar prophage induction phenotype to the $\lambda r1$ single mutant (data not shown). Therefore, the idea that Cro binding to O_{R3} is important in prophage induction, although plausible, lacks any supportive evidence. We are currently constructing phage mutants defective in Cro binding at

O_{R3} but with normal CI binding in order to test this proposal.

The effects of the O_{R3} mutations on switching from lysogeny are instead readily explained by the degree to which CI is able to limit its own concentration in the lysogenic state; the inducibility of the phage decreased with increasing lysogenic CI concentration. This is not surprising, because alterations in CI levels will affect the duration and intensity of RecA co-protease activity (dependent on the dose of DNA damage) that is needed to reduce the CI concentration to the lytic transition threshold (Bailone et al. 1979).

Negative autoregulation

An alternative way for λ to limit the CI concentration in the lysogen would be by reducing the degree of activation of P_{RM} by CI. What advantage is there, then, in using negative autoregulation? λ lysogeny is extremely stable. The apparent rate of spontaneous transition from the lysogenic state to the lytic state (in a $recA^-$ host) is of the order of 4×10^{-7} per generation (Little et al. 1999), or about once every 160 yr for a single cell. Despite this stability, lysogeny is also sensitive. Transient SOS-inducing signals are able to cause the prophage to efficiently and rapidly switch to lytic development. We believe that negative autoregulation allows λ to limit the lysogenic CI concentration to provide sensitivity without overly compromising the stability of the lysogen.

The regulatory consequences of negative autoregulation are illustrated in Figure 6, which shows how the CI concentration affects both the rate of gain of CI concentration and the rate of loss of CI concentration. CI has a half-life of >10 h in the cell (Parsell et al. 1990); the loss of CI concentration is primarily by dilution due to cell growth. Exponential cell growth means that CI concentration decays with a constant half-life, that is, that the rate of loss of CI concentration is first order, or proportional to the CI concentration (shown in Fig. 6 as a dashed line). Assuming that our measured P_{RM} activities are proportional to the rate of gain of CI concentration, we can plot gain curves for CI either with (*wt*) or without ($r1$) negative autoregulation (Fig. 6, solid lines). Changes in the concentration of CI are dictated by the relative rates of gain and loss of the protein: if gain is greater than loss, the CI concentration will increase; if gain is less than loss, the CI concentration will decrease. The lysogenic CI concentration in each case is the stable state of this regulatory system, where gain of CI balances loss of CI. With the removal of negative autoregulation ($r1$), the stable state occurs at a much higher CI concentration, as observed. However, negative autoregulation is not the only way to achieve the wild-type lysogenic CI concentration—a reduction in the degree of positive autoregulation would have the same effect (Fig. 6, dotted curve). The advantage of negative autoregulation is that it provides extra stabilization of the lysogenic CI concentration against fluctuations (Becskei and Serrano 2000). From Figure 6, it can be seen that any downward fluctuation in CI concentration will be resisted more

Figure 6. The function of negative autoregulation. The curves show the rate of gain of CI concentration versus its concentration for λ wt and λ r1 (solid lines). The first order rate of loss of CI concentration is plotted on the same axes as a straight line passing through the origin (dashed line). Note that the y-axis represents the rate of gain or the rate of loss of CI concentration, as indicated on the curves. The stable steady state CI concentrations in the λ wt and λ r1 lysogens (1 and 2.4 CI wild-type lysogenic units [WLU]) occur where the rates of CI gain and loss are equal. The dotted gain curve shows that the wild-type CI concentration could be achieved without negative autoregulation, but this would result in a less stable lysogenic CI concentration. Without negative autoregulation, a downward fluctuation in the CI concentration (e.g., to 0.5 WLU) causes the system to respond with a small net gain of CI (gain – loss = *a*) and therefore a fairly weak restoration of the CI concentration. With negative autoregulation (wt), the same fluctuation causes the system to produce a larger net gain of CI (gain – loss = *a* + *b*) due to derepression of P_{RM} . This gives a stronger restoration of the CI concentration.

strongly in the presence of negative autoregulation because the resulting net rate of gain of CI (the difference between its rates of gain and loss; *a* + *b* in Fig. 6) will be greater than in the absence of negative autoregulation (*a* in Fig. 6). This noise reduction system makes it less likely that random fluctuations will lower CI concentrations to a point where lysogeny cannot be maintained.

We expect that the ability of negative autoregulation to confer stability and sensitivity will be used in the genetic switches that underlie development in more complex organisms.

Materials and methods

Strains and media

C600 was used as a λ indicator. NK7049 (Δ lacIZYA)X74 galOP308 Str^R Su⁻ from R. Simons (Simons et al. 1987) was the host for all LacZ assays. DH5 α was used for recombinant DNA work. DH5 α Z1 (Lutz and Bujard 1997) was the *lacI*^q host for construction of the *ci* and *cro* gene expression plasmids. λ^+ and 434 were obtained from the laboratory of Dale Kaiser at Stanford University. λ att80 was λ imm^h ϕ 80att ϕ 80 from Gary Gussin (University of Iowa, Iowa City). λ RS45 (Simons et al. 1987) was

modified for *lac* operon fusions. λ lysogens were tested for monolysogeny by the polymerase chain reaction (PCR) method of Powell et al. (1994); the λ att80 lysogens were checked by an analogous test. Cells were grown in LB (Miller 1972) with addition of ampicillin (30 μ g/mL for pZC320, 100 μ g/mL for pZE15) and kanamycin (50 μ g/mL for pUHA1).

Construction of λ O_R3 mutants

The *r1* and *c12* mutations were introduced by the Quickchange oligonucleotide-directed method (Stratagene) into pDT843, a pACYC184 derivative carrying the λ^+ *Bcl*I:37352–*Eco*RV:39355 fragment. The sequence of the *Bcl*I:37352–*Bst*XI:38292 region was then confirmed and this fragment was used to replace the equivalent wild-type portion of pAP831, a pACYC184 derivative carrying the λ^+ *Nhe*I:34678–*Eco*RV:39355 fragment. The wild-type and mutant sequences were crossed onto the λ chromosome by infecting C600 cells carrying pAP831 or its mutant derivative with λ imm⁴³⁴ and selecting imm⁺ recombinants among the progeny as plaques on C600(434). Recombination leads to replacement of the imm⁴³⁴ region with λ sequences. The sequence of the *Bcl*I:37352–*Bst*XI:38292 region of the λ isolates was confirmed.

Phage infection and prophage induction

For phage infection, log phase cultures in LBM (LB, 10 mM MgSO₄) plus 0.2% maltose were concentrated fivefold by centrifugation and infected at a multiplicity of addition $\sim$ 0.1 at 37°C for 10 min. The culture was diluted at least 100-fold in LB, incubated with shaking at 37°C, and plaque-forming units (PFU) assayed over time by mixing dilutions with indicator bacteria (C600 in LBM) and 3 mL molten top agar (0.7% agar, 10 mM MgSO₄) and pouring onto TB plates (1% Bacto-tryptone [Difco], 0.5% NaCl, 1.5% Bacto-agar). PFU/mL were normalized to pre-infection colony forming units (CFU)/mL, assayed by spreading on TB plates. For UV irradiation, log phase cultures in LB were diluted in M9 salts (Miller 1972) and irradiated with a germicidal UV lamp, all manipulations being performed under yellow lighting at 37°C. The kinetics of phage production after UV were assayed as for infection. To measure the fraction of lysogens induced after UV, we assayed aliquots of the cultures for PFU with NK7049 in LBM on LB plates. Preirradiation CFU/mL were assayed by pouring onto LB plates with top agar.

Immunoquantitation of CI

Gel electrophoresis and Western blotting were essentially as described by Sambrook et al. (1989). Cells were grown at 37°C in LB, plus ampicillin, plus kanamycin (plus IPTG, as appropriate) to O.D.₆₀₀ $\sim$ 0.6, washed, resuspended in 1/200th volume (adjusted for slight deviations from O.D.₆₀₀ = 0.6) in MinA (Miller 1972), sonicated, diluted 1:1 with 2 $\times$ SDS sample buffer, and heated to 95°C for 2 min. After SDS-PAGE (10% polyacrylamide), samples were electrotransferred to PVDF membranes (0.1 μ m pore, MSI, Westborough, MA). Membranes were blocked and washed in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% low fat milk, and 0.1% Tween 20. Primary rabbit antibody to λ CI was from Invitrogen and in some experiments was preadsorbed to nonlysogenic cell extract in blocking buffer. HRP-conjugated goat anti-rabbit secondary antibody and Super-Signal reagents for chemiluminescence were from Pierce. A range of exposures of Kodak Max film were scanned by laser densitometry and analyzed using NIH Image 1.62.

Gel shift quantitation of CI

Extracts were prepared from 80 mL of log phase culture at 37°C in LB, plus ampicillin, plus kanamycin (plus IPTG, as appropri-

ate) at $O.D._{600} \sim 0.6$, washed, and concentrated 100-fold in TEG150 (50 mM Tris-HCl at pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 10% glycerol), followed by sonication and centrifugation at 5000g to remove debris. For the gel mobility shift reactions, extracts were diluted equally in TEG150 and then the CI-containing extracts were diluted to differing extents using diluted extract from nonlysogenic cells, that is, all reactions contained the same amount of cellular extract but different amounts of CI. The DNA probe was a gel-purified 154 bp PCR-generated $O_L P_L^-$ fragment from pRS308 ΔYAO_L , labeled by treatment of one primer with [γ - 32 P]ATP and T4 polynucleotide kinase. Binding reactions (10 μ L) were in TEG150 at 0°C with ~ 150 pM probe DNA and 4 ng/ μ L sheared salmon sperm DNA as competitor. After 30–50 min, 4 μ L of each reaction was loaded onto a 6% polyacrylamide, 10% glycerol, 1 $\times$ TBE gel at 4°C. Loading and running were done at 22 mA/cm²; the running buffer was 1 $\times$ TBE (Sambrook et al. 1989). After drying of the gel, the total and unbound DNA in each lane were quantitated using storage phosphor technology (Molecular Dynamics) and the fraction of DNA bound calculated as 1 – unbound/total.

Construction of chromosomal $P_{RM}::lacZ$ reporter fusions

The 37880–38064 P_{RM} region of λ was amplified by PCR with primers flanked by *Hind*III and *Xho*I sites from pDT843wt or its *r1* and *c12* derivatives, and inserted into the polylinker of the *lacZ* reporter plasmid pTL61T (Linn and St. Pierre 1990). The $P_{RM}::lacZ$ fusions were then recombined in vivo onto λ RS45 ΔYA and λ RS45 ΔYAO_L (see below) by infection of plasmid-containing strains followed by screening for blue plaque forming phage on plates containing X-gal (Simons et al. 1987). The resulting λ RS45 ΔYA - $P_{RM}::lacZ$ and λ RS45 ΔYAO_L - $P_{RM}::lacZ$ phage (wt, *r1*, and *c12*) were used to make monolysogens of NK7049.

The modified *lacZ* reporter phage λ RS45 ΔYA and λ RS45 ΔYAO_L used earlier were derived from the *lac* reporter λ RS45 (Simons et al. 1987) by deleting the DNA between the *Sgr*AI site (in *lacY*) and the *Bsr*GI site (in *lacA*) to inactivate these genes (Fig. 4A). This was done to avoid IPTG-controlled expression of CI being affected by *lacY* expression from the reporter, because the LacY permease aids uptake of IPTG by the cell. The ΔYA deletion was first made in pRS308 (Simons et al. 1987) by endfilling and religating the *Sgr*AI and the *Bsr*GI sites and was then recombined in vivo onto λ RS45 $P_{tet}::lacZ$ to obtain λ RS45 ΔYA . Recombinant phage were isolated as white plaque formers on X-gal (λ RS45 $P_{tet}::lacZ$ gives blue plaques) and then checked for the presence of ΔYA using PCR. To make λ RS45 ΔYAO_L , a synthetic (sticky-ended) $\lambda O_L P_L^-$ sequence containing the O_L1 , O_L2 , and O_L3 operators (underlined) and mutations to inactivate P_L (lowercase), GTAcATATCACCGC-CAGTGGTATTTActaCAACACCGCCAGAGATAATTTATCACCGCAGATGGTTATCGCCGG, was inserted into the *Sgr*AI and *Bsr*GI sites (right end toward *lacZ*) in pRS308 and recombined onto λ RS45 $P_{tet}::lacZ$ as for λ RS45 ΔYA . The relevant sequences of λ RS45 ΔYA and λ RS45 ΔYAO_L were confirmed.

Construction of CI expression plasmids

The wild-type λ CI gene (9 bp upstream of start codon to the stop codon) was amplified by PCR using primers with flanking *Pst*I and *Hind*III sites and inserted into pBS-SK+ (Stratagene) to give pBS-SK+CI. To make pZC320CI, the *Pst*I–CI–*Bam*HI fragment from pBS-SK+CI was inserted downstream of P_{lac}^+ ; between the *Nsi*I and *Bam*HI sites of the mini-F based, ampicillin-resistant plasmid pZC320 (Shi and Biek 1995). The colE1, ampicillin-resistant expression plasmid pZE15 was derived by inserting

P_{lac}^+ from pZC320 (on an endfilled *Bsa*BI–*Bam*HI fragment) into the endfilled *Xho*I and *Xba*I sites of pZE12-*luc* (Lutz and Bujard 1997), replacing the $P_{LlacO-1}$ promoter and *luc* gene. The pZE15CI or pZE15cro expression plasmids were made by inserting the CI or cro genes between the *Nsi*I and *Bam*HI sites downstream of P_{lac}^+ in pZE15: CI by inserting the *Pst*I I–*Bam*HI fragment from pBS-SK+CI; cro on a PCR-generated fragment (16 bp upstream of start codon to the stop codon with flanking *Pst*I and *Bam*HI sites). The sequences of the cloned CI and cro genes were confirmed. Lac repressor was supplied to the expression plasmids by pUHA1, a p15A plasmid encoding kanamycin resistance and carrying the wild-type *lacI* gene and promoter, obtained from H. Bujard.

LacZ assays

Kinetic LacZ assays were done in 96-well microtiter plates by an extensively modified method of Miller (1972). Fresh colonies on selective LB plates were resuspended in LB and used to inoculate 200 μ L of LB + antibiotic + IPTG. Dishes were sealed and incubated for ~ 16 h without shaking. These cultures were subcultured by diluting 2 μ L into 98 μ L fresh medium and incubated with rotation to an $O.D._{600}$ of 0.2–1.2 (log phase). OD_{600} was measured using a Labsystems Multiskan Ascent plate reader with a 620-nm filter; the $O.D._{620}$ values were converted to $O.D._{600}$ (1-cm path length) values using an empirically derived relationship and adjusted for light-scattering nonlinearity according to Bipatnath et al. (1998). Cells were chilled and then permeabilized with polymyxin B (Schupp et al. 1995) by adding 25 μ L of culture plus 25 μ L of LB to 150 μ L of lysis buffer in a microtiter dish. Lysis buffer was TZ8 (100 mM Tris-HCl at pH 8.0, 1 mM MgSO₄, 10 mM KCl) + 2.7 μ L/mL 2-mercaptoethanol and 50 μ g/mL polymyxin B. The presence of detergents and chelating agents (Schupp et al. 1995) did not improve the assay. Use of pH 8 for the assay buffer rather than the pH 7 used by Miller (1972) improved display of *o*-nitrophenol in the absence of Na₂CO₃ added to stop the reaction. Assays were at 28°C and were begun by addition of 40 μ L of *o*-nitrophenyl- β -D galactoside (4 mg/mL in TZ8). The plate reader was used to incubate the reactions and take A_{414} readings every 2 min for 1 h. Enzyme activity was determined as the slope of the line of best fit of A_{414} versus time (readings with $A_{414} > 2.5$ were ignored). Enzyme activity was found to be directly proportional to the $O.D._{600}$ of the culture and the volume of culture added to the assay (V, in μ L). LacZ units were calculated as $200,000 \times (A_{414}/\text{min}) / (A_{600} \times V)$ and were roughly equivalent to standard Miller units.

Acknowledgments

We thank Donald Biek, Herman Bujard, Gary Gussin, Thomas Linn, and Bob Simons for gifts of plasmids, phage, and bacterial strains; Don Court, Alex Gann, John Little, Mark Ptashne, John Reinitz, and Kim Sneppen for discussions and comments on the manuscript; Keith Shearwin and other members of the Egan lab for advice and support; and John Little for helping us correct one of our constructs. Research in the Egan lab is funded by the Australian Research Council. This paper is dedicated to Mark Ptashne and his colleagues, whose seminal work on λ has been an inspiration.

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Octamerization of λ CI repressor is needed for effective repression of P_{RM} and efficient switching from lysogeny

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Genes Dev. 2001, **15**:

Access the most recent version at doi:[10.1101/gad.937301](https://doi.org/10.1101/gad.937301)

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