Cro’s role in the CI–Cro bistable switch is critical for λ’s transition from lysogeny to lytic development

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CI represses cro; Cro represses ci. This double negative feedback loop is the core of the classical CI–Cro epigenetic switch of bacteriophage λ. Despite the classical status of this switch, the role in λ development of Cro repression of the PRM promoter for CI has remained unclear. To address this, we created binding site mutations that strongly impaired Cro repression of PRM with only minimal effects on CI regulation of PRM. These mutations had little impact on λ development after infection but strongly inhibited the transition from lysogeny to the lytic pathway. We demonstrate that following inactivation of CI by ultraviolet treatment of λ lysogens, repression of PRM by Cro is needed to prevent synthesis of new CI that would otherwise significantly impede lytic development. Thus a bistable CI–Cro circuit reinforces the commitment to a developmental transition.

[Keywords: Bistability; epigenetic; bacteriophage λ; genetic switch; Cro; transcriptional control]

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Bacteriophage λ, with its ability to choose between lytic and lysogenic modes of development, has provided an important model system for aiding understanding of the gene regulatory mechanisms and strategies that underpin cell differentiation in more complex organisms (Kauffman 1973; Herskowitz and Hagen 1980; Ptashne 2004; Court et al. 2007; Murray and Gann 2007). Since the late 1960s, it has been known that a portion of the λ genome, encoding the CI and Cro repressors and the promoters they regulate [Fig. 1A], comprises a bistable switch—a gene control circuit that is able to exist stably in either of two distinct, self-sustaining regulatory states: “immune” and “anti-immune” [Eisen et al. 1970; Neubauer and Calef 1970; Toman et al. 1985; Svenningsen et al. 2005]. In the immune (CI-dominant) state, the CI protein is expressed from the lysogenic promoter PRM and binds cooperatively to two operators, O−1 and O−2, to repress the PR lytic promoter and thus block transcription of the cro gene. CI simultaneously activates transcription of its own gene from PRM. In the anti-immune (Cro-dominant) state, Cro is expressed from PR and prevents CI expression, presumably by virtue of its high affinity for O+3, where it can bind to repress PRM (Johnson et al. 1978; Takeda 1979; Meyer et al. 1980). This CI–Cro double negative feedback loop, augmented by direct CI positive feedback, provides stable and heritable alternative epigenetic states. Understandably, it has been tempting to assume that the lytic mode of λ development is dependent on the anti-immune state of this switch. For example, in Lewin (1999) it is stated that “Cro...prevents synthesis of the repressor (a necessary action if the lytic cycle is to proceed).” However, although the immune state of the switch is necessary for lysogeny, it is not clear what are the roles of anti-immune state of the switch and the repression of PRM by Cro during λ lytic development.

Cro is essential for λ lytic development, but a large body of indirect evidence indicates that it is Cro’s action in turning down lytic transcription, rather than its repression of PRM, that is critical for lytic development after infection. Cro binds with highest affinity to O+3 but it also binds to O−1 and O−2 at PR and to the operators at PR [Johnson et al. 1978; Takeda 1979; Darling et al. 2000], from where it can exert at least a two- to fourfold turn-down of early lytic transcription (Eisen et al. 1970; Adhya and Gottesman 1982; Svenningsen et al. 2005). Thus, Cro reduces the expression of every lytically expressed gene, including the regulatory genes N, cII, cII, and Q; λ ci’ cro” mutants show a severe defect in lytic development that has been attributed to either an extremely high frequency of lysogenization after infection [Eisen et al. 1970] or a stalling of lytic development without causing increased lysogeny [Folkmanis et al. 1977]. In either case, the defect appears not to be due to loss of Cro repression of PRM but instead results from...
overexpression of the CII and CIII proteins, as it can be suppressed by cIIΔ and cIIIΔ mutations [Folkmanis et al. 1977]. CII, stabilized by CIII, activates an alternative promoter for cl, P RE. CII also activates the P1 promoter for the integrase protein that inserts the λ genome as a prophage into the host chromosome, and the P AOQ promoter that inhibits the production of the late gene activator Q [Kobilier et al. 2005; Court et al. 2007]. Thus high activity of CII in cro− phages should enhance lysogenization and inhibit lytic development. Because of these major pleiotropic effects of cro− mutations, it has not been possible to determine whether Cro repression of P RM is important in the choice between lysis and lysogeny or in the progression of lytic development after infection by cro− phages.

It seems more likely that Cro repression of P RM is important during prophage induction, the transition from lysogeny to lytic development that is initiated by RecA-stimulated cleavage of CI following DNA damaging treatments such as ultraviolet (UV) irradiation [Bailone et al. 1979; Little 1984]. In this situation, CI expression should be more strongly dependent on P RM than CII-activated P RE for two reasons. First, production of CI from P RE appears to be low during prophage induction, in part because CII production is reduced by the SOS-induced OOP antisense RNA [Krinke et al. 1991]. In support of this, it has been shown that P RE− mutations do not affect spontaneous phage production from a lysogen [Baek et al. 2003]. Second, RecA activity is not always high enough and sustained enough to remove all of the CI present in the lysogen [Bailone et al. 1979], leaving residual CI that should activate P RM and lead to more CI production. It has been proposed that Cro repression of P RM may be needed to prevent recovery of CI levels and re-establishment of repression of the lytic promoters once the RecA signal decays [Johnson et al. 1981].

However, experimental evidence regarding the role of Cro repression of P RM in prophage induction is unclear. Initial experiments showed poor UV induction of a phage bearing mutations in OΔ3 that reduced Cro repression of P RM [Johnson 1980]. But it has since been shown that the poor prophage induction in this mutant was due to a lack of CI binding to OΔ3, which results in a threefold increase in lysogenic CI levels that is inhibitory to prophage induction [Dodd et al. 2001]. Although Svenningsen et al. (2005) found little effect of Cro on P R derepression, interpretation of their experiments is complicated by the use of temperature inactivation of CI and a cro− mutant. Atsumi and Little (2006) created highly modified λ phages in which Cro is substituted by the lac repressor, which was able to repress the lytic promoters but not P RM. It was concluded that Cro repression of P RM is not needed for lytic development and plays only a modulatory role in prophage induction. However, this study is weakened by the lack of a comparison with similarly manipulated phages in which lac repressor is able to repress P RM [see Discussion].

We sought to address these uncertainties in this important model bistable system by clarifying the role of Cro repression of P RM and, by extension, the role of the bistability of the CI–Cro switch in normal λ development. Our approach was to create and characterize operator mutations that specifically inactivated Cro repression of P RM and to measure the effect of these mutations in a wild-type λ background. We found that these mutations had mild effects on the choice between lytic and lysogenic development and on the efficiency of lytic development after infection, but strongly impaired prophage induction by UV, indicating a critical role of Cro repression of P RM in the transition from lysogenic to lytic development. Studies of a cl−cro switch reporter construct confirmed loss of the bistability of the switch due to elimination of the anti-immune state and indicated that Cro repression of P RM is necessary to prevent recovery of CI after UV induction.

Results

Cro repression of P RM

The ability of Cro to strongly repress transcription from P RM [Meyer et al. 1980] was confirmed previously, using a P RM−lacZ reporter with Cro expressed from a multicopy plasmid [Dodd et al. 2001]. To gain some appreciation of the extent of repression by Cro that P RM might experience at early times after infection of a cell by a single λ phage or after prophage induction, we compared the activities of chromosomal P RM−lacZ transcriptional reporters that produce either wild-type Cro or a mutant Cro protein in cis from P R [lacZ,P RM,P R,cro−] [Fig. 1B]. Under steady-state conditions, Cro expression from P R
in single copy is sufficient to turn $P_R$ down by ~50% (Svenningsen et al. 2005; A. Palmer, unpubl.). In our reporters, $P_{RM}$ was repressed at least 10-fold by Cro, from 38 to 3.6 LacZ units [Fig. 1B]. Our experience with transcriptional fusions is that there is usually a background LacZ level that is partly reporter vector-dependent [~2.5 U for this vector] and partly insert-dependent. Using a 2.5-U background gives an estimate of Cro repression of $P_{RM}$ of >30-fold [35.5/1.1]. We also measured the impact of single-copy Cro on the expression of CI protein from $P_{RM}$ by using a translational fusion, in which the first 20 codons of the $cI$ gene are fused to the lacZ gene [Fig. 1C]. LacZ activity in the absence of Cro was 5.6 U and was completely abolished by Cro [Fig. 1C]. We conclude that, in the absence of CI, Cro produced from a single-copy cro gene expressed from $P_R$ can completely abolish CI expression from $P_{RM}$ in cis. We expect that the weaker repression calculated for the transcriptional fusion is due to the non-$P_{RM}$ contribution to LacZ expression being >2.5 U.

**$O_R$ mutants defective in Cro repression of $P_{RM}$**

We wished to create mutations that eliminated Cro repression of $P_{RM}$ but had minimal other effects on the intrinsic activities of the $P_{RM}$ and $P_R$ promoters and their regulation by CI and Cro. We initially focused on $O_{R3}$ because previous reporter studies (Meyer et al. 1980) had indicated that Cro represses $P_{RM}$ when bound to $O_{R3}$ but not when bound to $O_{R2}$ and $O_{R1}$ [Fig. 2A]. Although CI and Cro recognize the same operators, binding site mutants that differentially affect Cro or CI binding can be obtained as the proteins recognize different bases within the operators. For example, we found previously that the $O_{R3}$-t1 mutation [Fig. 2A] eliminated CI repression but had no effect on Cro repression of $P_{RM}$ [Dodd et al. 2001]. To design the Cro-specific mutants, we were guided by exhaustive studies of the effects of base-pair changes in $O_{R3}$ on CI and Cro binding [Sarai and Takeda 1989; Takeda et al. 1989].

**Removing Cro repression**

Many multiple base-pair changes in $O_{R3}$ were investigated, but no combination completely eliminated Cro repression of $P_{RM}$ when Cro was supplied from a plasmid. To identify the source of this remaining repression of $P_{RM}$ we endeavored to “knock out” Cro binding to $O_{R3}$ by changing 9 base pairs [bp] of the 17-bp operator site [Fig. 2A]. Surprisingly, this $O_{R3}$-KO mutant displayed considerable residual repression by plasmid-supplied Cro [Fig. 2B], which we therefore suspected was due to Cro binding to $O_{R2}$. We confirmed this by making multiple mutations to create an $O_{R3}$-2-KO mutant [Fig. 2A]. It was only when the $O_{R3}$-2-KO and $O_{R3}$-KO mutations were combined that Cro repression of $P_{RM}$ was eliminated [Fig. 2B]. These results show that Cro can repress $P_{RM}$ by binding to either $O_{R3}$ or $O_{R2}$ [see Supplemental Material for further discussion].

![Figure 2](https://example.com/figure2.png)

**Figure 2.** $O_R$ mutations relieving Cro repression of $P_{RM}$. [A] The $P_{RM}$-$O_R$-$P_R$ region and the mutations used in this study. The c21, r1, r314A, c12, and r204 mutations change the $\Delta G$ of Cro binding to $O_{R1}$ by +1.7, +0.4, +1.7, +1.3, and +1.4 kcal/mol, respectively [Takeda et al. 1989], and the $\Delta G$ of CI binding to $O_{R1}$ by -0.2, -2.9, +1.0, +0.8, and +0.3 kcal/mol, respectively [Sarai and Takeda 1989]. [B] Activity of the $P_{RM}$ lacZ reporter (croA21 as in Fig. 1B) carrying KO mutations in $O_{R3}$ and $O_{R2}$, in response to Cro supplied from an IPTG-inducible expression plasmid [or an empty vector, dashed curve]. LacZ activities have been normalized to aid comparison of repression with the $O_{R3}$-2-KO mutation, which decreases $P_{RM}$ intrinsic activity by approximately twofold. [C] Effect of $O_R$ mutations on repression of $P_{RM}$ by Cro expressed from a single-copy cro gene in cis. The $P_{RM}$ lacZ reporters are as in Figure 1B, except that $\Delta$cro here indicates truncation of cro at the +43 position of $P_R$. Fold repression is calculated without subtraction of background. [B, C] Errors are 95% confidence intervals.
We were unable to find a combination of \(O_{R}\)2 and \(O_{R}\)3 mutations that eliminated repression of \(P_{RM}\) by Cro expressed from a plasmid, without also strongly affecting intrinsic \(P_{RM}\) activity or its activation by CI. However, we reasoned that resistance to a high level of Cro may be an unnecessarily stringent test for the mutations. We therefore measured \(P_{RM}\) repression by Cro for the three most promising mutants in the more natural early lytic situation where a single-copy \(cro\) gene is expressed by \(P_{R}\) in \(cis\) to \(P_{RM}\) [Fig. 2C]. The \(O_{R}\)3-x3 mutant combines three mutations at \(O_{R}\)3 [Fig. 2A] and, like the \(O_{R}\)3-KO mutant, gave only weak repression of \(P_{RM}\) by Cro in \(cis\). The addition of the \(O_{R}\)2-r204 change to the \(O_{R}\)3-x3 mutant, to give the \(O_{R}\)2/3-x4 mutant, eliminated \(P_{RM}\) repression at this Cro concentration [Fig. 2C]. Thus, these mutants seemed to be sufficiently defective in Cro repression of \(P_{RM}\) for our purposes.

**Additional effects of the mutations**

We further examined the above mutations for effects on regulation of \(P_{RM}\) and \(P_{R}\). Our initial testing had suggested that the \(O_{R}\)3-x3 mutation had small effects on regulation of \(P_{RM}\) by CI. Although the \(O_{R}\)2/3-x4 mutation appeared to cause a larger alteration in CI regulation, we examined it further because Cro repression of \(P_{RM}\) is more defective in this mutant [Fig. 2C]. We also tested the \(O_{R}\)3-KO mutation further. We expected this mutation to strongly reduce CI repression of \(P_{RM}\) and therefore included the \(O_{R}\)3-r1 mutant as a control that also eliminates CI repression of \(P_{RM}\) but does not affect basal \(P_{RM}\) activity or its repression by Cro [Dodd et al. 2001].

**Intrinsic \(P_{RM}\) activity** Figure 2C shows that in the absence of Cro expression \([cro]^{-}\), the \(O_{R}\)3-x3 mutation slightly increased the intrinsic activity of \(P_{RM}\) \([-1.3\text{-fold}]\), while the \(O_{R}\)2/3-x4 mutation increased it somewhat more \([-1.7\text{-fold}]\). Surprisingly, the \(O_{R}\)3-KO mutation did not change basal \(P_{RM}\) activity.

**Regulation of \(P_{RM}\) by CI** Figure 3A shows the response of \(P_{RM},\text{lacZ}\) reporters carrying the \(O_{R}\) mutations to a range of CI levels supplied by an isopropyl thio-\(\beta\)-D-galactoside (IPTG)-controlled expression plasmid. The wild-type \([WT]\) and \(O_{R}\)3-x3 mutant showed quite similar profiles: activation at low CI concentrations due to CI binding at \(O_{R}\)2, followed by repression at higher CI concentrations as \(O_{R}\)3 becomes occupied. CI repression of \(P_{RM}\) in the \(O_{R}\)3-x3 mutant was slightly stronger, suggesting that CI may bind slightly better to \(O_{R}\)3-x3. Despite its higher basal activity, \(P_{RM}\) in the \(O_{R}\)2/3-x4 mutant was only \(-75\%\) as active as wild-type \(P_{RM}\) over a range of CI concentrations, suggesting that the r204 mutation interferes with CI activation of \(P_{RM}\). The \(O_{R}\)3-KO mutation showed the same response of \(P_{RM}\) to CI previously described for the \(O_{R}\)3-r1 mutant [Dodd et al. 2001].

**\(P_{R}\) activity and its regulation by CI and Cro** Figure 3, B and C, shows the response of \(P_{R},\text{lacZ}\) reporters, carrying the \(O_{R}\) mutations, to Cro or CI supplied from plasmids or to CI supplied by a \(\lambda\) prophage. Although there are some small reproducible effects of the \(O_{R}\)3 and \(O_{R}\)2 mutations, the basal activity of \(P_{R}\) and its regulation by CI and Cro remained essentially the same. There was evidence for a slight reduction of Cro repression of \(P_{R}\) in the \(O_{R}\)2/3-x4 mutant [Fig. 3C], consistent with weakening of Cro binding to \(O_{R}\)2 [Meyer et al. 1980]. Inciden-
tally, this is possibly the first demonstration that Cro binding to O₈₃ does not affect P₈.

**Translation of cI from P₈ mRNA** The O₈ mutants lie just upstream of the cI start codon, and we realized that they could well affect ribosome binding for cI translation from the P₈ mRNA ([Fig. 2A]). The cI mRNA produced from P₈ begins at the cI start codon and therefore is not affected by the O₈ mutations. To measure this potential effect, we constructed the translational reporters shown in Figure 3D, in which cI codon 20 was fused to the lacZ reading frame and the λ P₇ promoter was substituted by the constitutive pb promoter from bacteriophage 186 (Kalions et al. 1986). The cro gene and P₈ promoter were mutationally inactivated. The wild-type construct gave 56 LacZ units, of which up to ~5.6 LacZ units could be contributed by translation of the P₈ transcript (see Fig. 1C). The O₈-3-x3, O₈-3-r1, and O₈-2/3-x4 mutations had relatively small effects on cI translation from the P₈ mRNA, but the O₈-3-KO mutation increased this translation sixfold. Although this drastic effect would seem to render the O₈-3-KO mutant useless for our comparisons, we show later that this effect does not have a large impact on the development of the phage.

In summary, our testing of a number of O₈-3 and O₈-2 mutants provided two pairs of mutants whose comparison was expected to be instructive with regard to the role of Cro repression of P₈. The major difference between O₈-WT and O₈-3-x3 is that the mutant retains only ~12% of P₈ repression by single-copy Cro [1.5/11.9]. However, the O₈-3-x3 mutation increases basal P₈ by ~1.3-fold and slightly increases CI repression of P₈. We included the O₈-2/3-x4 mutant as an adjunct to this pair because of its greater defect in Cro repression of P₈. However, O₈-2/3-x4 has a larger effect on basal P₈ activity (up 1.7-fold) and reduces CI-stimulated P₈ activity by ~25%.

The other pair of mutants, O₈-3-r1 and O₈-3-KO, differ in that O₈-3-KO retains only 15% of Cro repression of P₈ [1.8/11.9], while Cro repression of P₈ in O₈-3-r1 is normal. A caveat to the comparison of this pair is that translation of cI from the P₈ mRNA is approximately ninefold more efficient in λ.O₈-3-KO compared with λ.O₈-3-r1 [340/38] (Fig. 3D).

The four O₈ mutants were therefore transferred into the genome of λ.b::kan, hereafter referred to as λ.WT, which is an otherwise wild-type λ carrying an insertion of the kanamycin resistance gene into the nonessential b region. The resulting phages gave turbid plaques of normal appearance, and single lysogens were obtained without difficulty. We measured the ability of the O₈ mutant phages to complete lytic development after infection, to enter lysogeny after infection, and to be induced from lysogeny into lytic development.

**Effects of the mutations on lytic and lysogenic development after infection**

The O₈ mutations had only small effects on phage production after infection, confirming that Cro repression of P₈ is not essential for lytic development. Single step burst curves showed that the timing of phage production was similar for the wild type and the mutants [an example is given in Fig. 4A]. However, we found in repeated experiments that the average number of phages produced per infection (burst size) of the O₈-3-x3 mutant was slightly lower than wild type (75%) (Table 1). Similarly, the O₈-3-KO mutant burst size was 66% of λ.O₈-3-r1. The burst deficit did not increase with the further loss of Cro repression of P₈ in the O₈-2/3-x4 mutant.

The proportion of infected cells that formed lysogens was slightly increased in the mutants with defective Cro repression of P₈. The frequency of lysogeny of the O₈-3-x3 mutant was 30% higher than the wild type and was 25% higher for λ.O₈-3-KO compared with λ.O₈-3-r1 (Table 1). A larger increase in lysogenization was seen with λ.O₈-2/3-x4.

These results are consistent with Cro repression of P₈ causing a slight enhancement of lytic development and a slight decrease in lysogenization after infection, but alternative explanations are possible. In particular, for the O₈-3-KO mutant, these effects could be due to the large increase in the efficiency of CI production from P₈ mRNA (Fig. 3D).

We were somewhat surprised that such a large increase in CI production from a transcript that is necessary for efficient lysogenization, did not have a larger impact. Presumably, other factors, such as integrase expression from P₇, P₉, inhibition of Q, or cellular factors that determine whether CII is active at all, are limiting for lysogenization under our conditions.

**Effects of the mutations on prophage induction**

Because CI is expressed only from P₈ in the lysogen, altered CI regulation of P₈ changes the steady-state lysogenic concentration of CI, which can in turn affect the efficiency of prophage induction (Dodd et al. 2001; Michalowski et al. 2004). Western blotting of extracts of single lysogens ([Fig. 4B]) showed that the O₈-3-KO mutation or the O₈-3-r1 mutation caused a approximately threefold increase in lysogenic CI level, as expected due to lack of CI negative autoregulation. The O₈-3-x3 and the O₈-2/3-x4 mutant lysogens contained 72% and 57% of the wild-type CI level, respectively, consistent with their altered regulation of P₈ by CI ([Fig. 3A]). On the basis of these measurements alone, one would expect the O₈-3-x3 and the O₈-2/3-x4 mutant lysogens to be somewhat more easily induced than wild type and the O₈-3-KO mutant lysogen to be induced poorly, like the O₈-3-r1 mutant (Dodd et al. 2001).

The O₈ mutants were strongly defective in prophage induction after UV (one experiment is shown in Fig. 4C). Table 1 summarizes the prophage induction behavior of λ.WT or the O₈ mutants after two different doses of UV and also in the absence of inducing treatments [spontaneous phage production]. For the UV experiments, the fraction of lysogens that entered lytic development (that is, produced at least one phage) and the total number of phages produced per lysogenic cell, were measured, al-
Figure 4. Behavior of the $\lambda$R mutant phages. [A] Phage production after infection. C600 cells were infected at a phage:bacterium ratio $<0.01$ with $\lambda$WT or $\lambda$R mutant phages and assayed for IC (lysing cells + FP) at various times. Values are normalized to preburst averages. [B] CI Western blotting of extracts from C600 nonlysogenic cells (non) or single C600 lysogens of $\lambda$WT or $\lambda$R mutant phages. [Note that there is a cross-reacting host band running just below CI]. Values are CI levels relative to wild type (with 95% confidence limits) obtained by quantitation of the blots ($\sigma = 8$ or 9). [C] Phage production after UV irradiation (10 J/m$^2$) of C600 lysogens. [D] Temperature induction of $\lambda$clts and $\lambda$clts.$\lambda$O$_R$3-x3 prophages. C600 lysogens were transferred from 30°C to 39°C and samples were assayed for FPs. [E] UV induction [as in C] of $\lambda$O$_R$3-r1 and $\lambda$O$_R$3-KO prophages and their $P_{RE}^+$ derivatives. For the $\lambda$O$_R$3-r1 $P_{RE}^+$ and $\lambda$O$_R$3-r1 $P_{RE}^-$ phages, respectively, 56% and 50% of lysogens were induced, producing 26 and 32 phage per irradiated lysogen. For the $\lambda$O$_R$3-KO $P_{RE}^+$ and $\lambda$O$_R$3-KO $P_{RE}^-$ phages, respectively, 18% and 30% of lysogens were induced, producing on average 0.6 and 0.9 phage per irradiated lysogen.

If the prophage induction defect of $\lambda$O$_R$3-x3 relative to $\lambda$WT is due to the loss of Cro repression of $P_{RM}$ and consequent overproduction of CI, then it should be relieved in the absence of CI activity. To test this, we replaced the cl$^+$ gene of $\lambda$WT and $\lambda$O$_R$3-x3 with the temperature-sensitive cl$_{K57}$ allele. Figure 4D shows that after induction of the clts lysogens by raising the temperature to 39°C, there was very little difference in phase production for the $\lambda$O$_R$3-x3 and wild-type lysogens (wild type and $\lambda$O$_R$3-x3 gave 259 ± 71 and 231 ± 33 phase per induced cell, respectively; $\pm SD$, $n = 2$). Clearly, the prophage induction defect caused by the $\lambda$O$_R$3-x3 mutation is dependent on the presence of active CI.

We expected that the poor prophage induction of the $\lambda$O$_R$3-KO mutant relative to the $\lambda$O$_R$3-r1 mutant was caused by increased CI production due to lack of Cro repression of $P_{RM}$ in the $\lambda$O$_R$3-KO mutant. However, the increased translation efficiency of cl from the $P_{RE}$ mRNA in the $\lambda$O$_R$3-KO mutant [Fig. 3D] could conceivably increase CI levels after induction and contribute to the defect. Transcription from the $P_{RE}$ promoter does not normally affect phase production from a lysogen [Back et al. 2003], but it might do so with a sixfold increase in its production of CI. If so, mutational inactivation of $P_{RE}$ should relieve the prophage induction defect of the $\lambda$O$_R$3-KO mutant. We found that introduction of a $P_{RE}^-$ mutation into the $\lambda$O$_R$3-r1 and $\lambda$O$_R$3-KO prophages had very little effect on phase production after a 10 J/m$^2$ UV dose, with the $\lambda$O$_R$3-KO $P_{RE}^-$ mutant retaining the induction defect relative to $\lambda$O$_R$3-r1 $P_{RE}^+$ [Fig. 4E]. Thus the defective prophage induction seen for the $\lambda$O$_R$3-KO mutant is not due to increased CI expression from $P_{RE}$ mRNA.
Role of Cro repression of $P_{RM}$

Table 1. Behavior of the mutant phages

| Phage property | $\lambda WT$ | $\lambda O_{R3}^{-}x3$ | $\lambda O_{R3}^{-}/x4$ | $\lambda O_{R3}^{-}r1$ | $\lambda O_{R3}^{-}KO$
|----------------|-------------|------------------------|----------------------|---------------------|-----------------
| **Phage production after infection** | | | | | |
| Burst size$^a$ | 265 ± 44 (10) | 204 ± 42 (3) | 207 ± 49 (3) | 270 ± 32 (3) | 165 ± 27 (4) |
| % Wild type$^b$ | 100 | 7 (6–66) | 7 (54–107) | 8 (74–108) | 5 (47–72) |
| % $O_{R3}^{-}r1$ | — | — | — | — | 100 |
| Establishment of lysogeny | — | — | — | — | 66 |
| **Frequency of lysogeny (%)** | 6.4 ± 0.6 (12) | 9.0 ± 2.0 (6) | 15.1 ± 2.7 (6) | 8.2 ± 3.2 (6) | 9.0 ± 1.8 (6) |
| % Wild type | 50 | 88 (100–120) | 120 (180–270) | 120 (100–140) | 150 (120–180) |
| % $O_{R3}^{-}r1$ | — | — | — | — | 100 |
| UV induction of prophage: 10 l/m$^2$ | — | — | — | — | 125 |
| **Fraction cells induced** | 1.09 ± 0.06 (6) | 0.74 ± 0.23 (5) | 0.98 ± 0.23 (4) | 0.52 ± 0.11 (6) | 0.17 ± 0.05 (6) |
| % Wild type | 100 | 68 (50–91) | 91 (70–118) | 47 (38–58) | 15 (12–19) |
| % $O_{R3}^{-}r1$ | — | — | — | — | 100 |
| Phage per lysogen | 255 ± 137 | 50 ± 32 | 55 ± 19 | 36 ± 26 | 0.7 ± 0.6 |
| Phage per induced cell$^c$ | 2.3 ± 120 | 69 ± 41 | 54 ± 9 | 66 ± 40 | 2.9 ± 1.4 |
| % Wild type | 50 | 32 (17–61) | 24 (12–49) | 27 (20–38) | 1.3 (1.2–1.4) |
| % $O_{R3}^{-}r1$ | — | — | — | — | 100 |
| **UV induction of prophage: 5 l/m$^2$** | — | — | — | — | 4.6 (3.3–6.6) |
| **Fraction cells induced** | 0.75 ± 0.19 (5) | 0.36 ± 0.10 (4) | 0.74 ± 0.16 (5) | — | — |
| % Wild type | 100 | 52 (38–71) | 93 (68–128) | — | — |
| Phage per lysogen | 88 ± 36 | 5 ± 4 | 15 ± 3 | — | — |
| Phage per induced cell | 117 ± 23 | 15 ± 19 | 16 ± 9 | — | — |
| % Wild type | 100 | 12 (5–29) | 39 (32–46) | — | — |
| Spontaneous phage production from lysogen | — | — | — | — | — |
| Phage per lysogen | 4·$10^{-5}$ (6) | 8·$10^{-5}$ (6) | 7·$10^{-5}$ (5) | 1·$10^{-5}$ (10) | 2·$10^{-5}$ (7) |
| % Wild type | 100 | 19 (11–33) | 22 (14–35) | 35 (25–50) | 5 (4–8) |
| % $O_{R3}^{-}r1$ | — | — | — | — | 100 |

$^a$Burst sizes are post-burst IC or FP values divided by the average of preburst IC values. Values are given ±95% confidence limits. Single numbers in parentheses show the number of experiments.

$^b$When a range of percentages are given in parentheses, the values in bold are averages of the ratios for $\lambda WT$ or $\lambda O_{R3}^{-}r1$ obtained within each experiment, and the ranges indicate the 95% confidence limits calculated using the logarithms of these ratios.

$^c$Values are averages of [Phage per lysogen/Fraction cells induced] in each experiment.

Effects of loss of Cro repression of $P_{RM}$ on the CI-Cro switch

We tested the effect of the $O_{R3}^{-}x3$ mutation on the isolated CI-Cro bistable switch, first, to confirm the long-held assumption that Cro repression of $P_{RM}$ is necessary for the anti-immune state of the switch and, second, to examine the kinetics of CI activity after UV induction in the absence of other regulatory $\lambda$ genes. We chose the $O_{R3}^{-}x3$ mutation as the best comparison with wild-type.

We measured $P_{R}$ transcription using chromosomal clts. $P_{RM}, P_{R}, P_{R-cro}, P_{R-cro}$ reporters that were $O_{R3}^{-}cro^{+}$ or carried the $O_{R3}^{-}x3$ and/or $cro^{-}$ mutations [Fig. 5A]. The reporters carry $O_{L}$, which is necessary for proper regulation of $P_{R}$ and $P_{RM}$ [Dodd et al. 2004], and include the $rexAB$ genes, which have no known role in CI-Cro regulation. After growth at 30°C, all four strains formed very pale blue colonies on Xgal plates, indicative of the immune state in which CI is produced and represses $P_{R}$ to give a very low LacZ expression. At 39°C all the strains formed dark blue colonies, due to inactivation of CI and derepression of $P_{R}$. When these dark blue colonies were restreaked at 30°C, most of the new colonies from the $O_{R3}^{-}cro^{+}$ strain were dark blue, displaying a persistent lack of CI repression of $P_{R}$ that is characteristic of the anti-immune state. In contrast, the restreaked colonies from the $cro^{-}$ or $O_{R3}^{-}x3$ strains were pale blue and had re-established the immune state. These results show that Cro repression of $P_{RM}$ is necessary for the anti-immune state of the CI-Cro switch.

To examine the kinetics of UV induction of the CI-Cro switch, we constructed $cl^{+}$ versions of the above reporters. Figure 5B shows a time course of LacZ production from $P_{R}$ after a 10 J/m$^2$ UV dose in the $O_{R3}^{-}cro^{+}$ and $O_{R3}^{-}x3$ $cro^{-}$ reporters. Figure 5C shows CI Western blotting from the same experiment. The two strains showed similar behavior in the early phase of induction, with some derepression of $P_{R}$ early after UV treatment [Fig. 5B, inset], before a sharp increase in $P_{R}$ activity after 20 min, in line with the substantial drop in CI levels evident in the Western blots.

In the later stages, the two strains behaved quite differently. In the $O_{R3}^{-}cro^{+}$ reporter, CI levels remained low for up to 4 h after induction, while LacZ levels continued to increase, though more slowly at later times, until reaching a maximum ~2 h after induction. The reduction in slope of the $O_{R3}^{-}cro^{+}$ LacZ curve with time is due in part to the accumulation of Cro, which partially
Figure 5. Effect of the O₃-3-x₃ mutation on UV induction of the CI-Cro switch. (A) Structure of the chromosomally integrated cI₃P₉0₂₃₄₅_P₉₃₄₅₆_cro₃₄₅₆_lacZ reporter constructs. LacZ activity from P₉₃₄₅₆, and Western blotting of CI [CI] following 10 J/m² UV treatment of the cro₃₄₅₆ reporters. (D) Relative LacZ activities in the O₃-3₃ and O₃-3-x₃ reporters after different UV doses. The strains carrying the croHTH⁺ mutation showed that the O₃-3-x₃ mutation made no difference to P₉₃₄₅₆ LacZ activity in the absence of Cro (open symbols, 10 J/m² UV treatment).

represses P₉₃₄₅₆ but also to the approach of LacZ accumulation to steady state, where the rate of LacZ production from P₉₃₄₅₆ is balanced by the rate of LacZ dilution due to cell growth (note that LacZ protein is stable and LacZ units are normalized to cell mass). The maximal activity of this P₉₃₄₅₆ reporter is expected to be ~180–220 U, reduced from the ~1150 U seen for P₉₃₄₅₆ in Figure 3, B and C, both by Cro repression [1.6-fold to twofold repression] [Svenningsen et al. 2005; A. Palmer, unpubl.] and by terminations at t₉₁ [approximately threefold reduction] [Fig. 5A; A. Palmer, unpubl.]. Thus, P₉₃₄₅₆ remained highly active in most cells of the O₃-3₃-cro⁺ strain for the duration of the experiment. In the O₃-3-x₃ cro⁺ reporter, the increase in LacZ units slowed in comparison with O₃-3₃-cro⁺ soon after 40 min and LacZ accumulation halted after 80 min, indicating that P₉₃₄₅₆ is substantially repressed at this point, consistent with the increased CI levels evident from the Western. After 100 min, LacZ activity slowly reduced, presumably as a result of dilution due to continued cell growth, indicating that P₉₃₄₅₆ is now strongly repressed and no new LacZ protein is being made. These results show that in the absence of Cro repression of P₉₃₄₅₆ CI activity becomes re-established after UV induction, reducing and eventually fully repressing P₉₃₄₅₆ activity.

The magnitude of the improvement in P₉₃₄₅₆ activity due to Cro repression of P₉₃₄₅₆ depends on the UV dose. Figure 5D shows the relative LacZ levels produced from P₉₃₄₅₆ in the presence compared with the absence of Cro repression of P₉₃₄₅₆ at three UV doses. At the high dose of 20 J/m², Cro repression of P₉₃₄₅₆ has relatively little effect on P₉₃₄₅₆ expression over the time of the experiment, presumably because RecA activity persists sufficiently to activate any CI made from P₉₃₄₅₆. At 5 J/m², the Cro/P₉₃₄₅₆ effect is two- to threefold stronger than that seen at 10 J/m². We expect that at low doses a larger fraction of CI remains after RecA activity has ended and can more readily restore CI levels by activation of P₉₃₄₅₆ unless the promoter is repressed by Cro.

We expect that Cro repression of P₉₃₄₅₆ is particularly important in prophage induction, in contrast to infection, because there will often be some activation of P₉₃₄₅₆ due to residual CI that has either escaped RecA inactivation or is produced from P₉₃₄₅₆ mRNA after RecA has disappeared. Thus, for Cro to impact on prophage induction, it must be able to repress P₉₃₄₅₆ in the presence of CI. To confirm this expected activity of Cro, we supplied varying levels of CI in trans to the lacZ/P₉₃₄₅₆-P₉₃₄₅₆-cro reporter of Figure 1B to see whether Cro produced in cis from P₉₃₄₅₆ could reduce P₉₃₄₅₆ activity in the presence of CI. Figure 6 shows that in the absence of Cro (the Δcro reporter), increasing IPTG induction of CI expression first activates then represses P₉₃₄₅₆ as seen previously [Dodd et al. 2001]. The substantial reductions in P₉₃₄₅₆ activity seen in the presence of Cro at lower CI concentrations dem-

Figure 6. Repression of P₉₃₄₅₆ by single-copy cro in cis in the presence of CI. The cro⁺ curve was obtained using the P₉₃₄₅₆-lacZ transcriptional reporter in Figure 1B. The reporter for the Δcro curve is the same except that the cro gene is truncated at the +45 position of P₉₃₄₅₆. CI was supplied with an IPTG-inducible expression plasmid system, with 150 µM IPTG producing a P₉₃₄₅₆ response equivalent to that at lysogenic CI levels [Dodd et al. 2001]. Errors are 95% confidence intervals. The dashed line shows the response of a P₉₃₄₅₆-lacZ (Δcro) reporter to CI (LacZ values divided by 5) [Dodd et al. 2004].
Demonstrate that Cro can indeed repress $P_{RM}$ in the presence of CI. The dashed line in Figure 6 shows how the activity of $P_R$, and thus the expression of Cro, is repressed by the increasing CI levels (Dodd et al. 2004). Remarkably, even when the single-copy $cro$ gene is substantially repressed by CI, the Cro protein produced can make a large difference to $P_{RM}$ activity. This result supports that idea that Cro expressed from a partially derepressed prophage can strongly inhibit CI production.

Discussion

The role of Cro repression of $P_{RM}$ after infection

Our results show that defective Cro repression of $P_{RM}$ does not dramatically affect lytic development or the establishment of lysogeny after infection by $\lambda$ phage. The $O_\mu R 3-x3$ mutation caused an $\sim 25\%$ decrease in burst size and an $\sim 25\%$ increase in lysogenization after infection, compared with wild type. A similar decrease in burst size ($33\%$) and increase in lysogenization ($25\%$) was seen with the $O_\mu R 3-KO$ mutation compared with the $O_\mu R 3-t1$ mutant. Although these slight increases in lysogenization and slight decreases in phage production are consistent with a lack of Cro repression of $P_{RM}$ altering the balance toward lysogenic development after infection, it is not possible to exclude other causes of these effects. The effect of the $O_\mu R 3-x3$ mutation might be due to the slightly increased basal $P_{RM}$ activity in this mutant ($30\%$ up compared with wild type), which could increase CI levels early in infection. Although there is no difference in basal $P_{RM}$ activity between the $O_\mu R 3-t1$ and $O_\mu R 3-KO$ mutants, the burst size and lysogenization changes in the $\lambda O_\mu R 3-KO$ mutant may be due to the mutant’s increased CI translation from $P_{RE}$ mRNA, which would be expected to favor lysogeny.

The role of Cro repression of $P_{RM}$ in prophage induction

In contrast, the effects of the $O_\mu R 3-x3$ and $O_\mu R 3-KO$ mutations on the transition from lysogeny to lytic development are substantial and can be directly attributed to the loss of Cro repression of $P_{RM}$. At a dose (10 J/m$^2$) that induced all $\lambda WT$ lysogens, phage production from $\lambda O_\mu R 3-x3$ lysogens was almost fivefold lower than wild type. Phage production from $\lambda O_\mu R 3-x3$ lysogens was 16-fold lower than wild type at the lower dose of 5 J/m$^2$, which induced 75% of $\lambda WT$ lysogens. At the 10 J/m$^2$ dose, 50% of $\lambda O_\mu R 3-t1$ lysogens were induced and phage production from the $\lambda O_\mu R 3-KO$ lysogens was 55-fold lower than for $\lambda O_\mu R 3-t1$. These effects are much stronger than can be explained by the other effects of the mutations. The $O_\mu R 3-x3$ mutation had a small effect on CI regulation of $P_{RM}$, but this should, if anything, assist induction, as it lowers the lysogenic CI concentration. The slight increase in basal $P_{RM}$ activity in $O_\mu R 3-x3$ (30%), which would tend to inhibit prophage induction, is likely to be insignificant compared with the eightfold increase in $P_{RM}$ activity due to loss of Cro repression. Apart from Cro repression of $P_{RM}$, the only difference between $\lambda O_\mu R 3-KO$ and $\lambda O_\mu R 3-t1$ is the increased CI translation from $P_{RE}$ mRNA in the $O_\mu R 3-KO$ mutant. However, we showed that this difference is not important in induction because inactivating $P_{RE}$ had no impact on prophage induction in these mutants. Thus, our results strongly support the Johnson-Ptashne conjecture (Johnson et al. 1981) that Cro repression of $P_{RM}$ is critical in prophage induction.

Comparisons with other studies

Our conclusions differ from two recent studies. Svenningsen et al. (2005) observed that the presence of the $cro^-$ gene on chromosomal $cl_{857} P_{RM} P_R crolacZ$ reporters did not increase $P_R$ activity at temperatures that partially released $P_R$ from repression by the temperature-sensitive mutant $Cl_{857}$ protein. However, the $cro^-$ mutation used to remove Cro repression of $P_{RM}$ also removes Cro repression of $P_R$, which would tend to mask any decreased $P_R$ activity caused by higher CI expression in this mutant. We believe that our experiments with Cro operator mutants, and with a transient SOS signal inactivating wild-type CI protein, more truly reflect the role of Cro repression of $P_{RM}$ in $\lambda$ prophage induction.

Atsumi and Little (2006) concluded that Cro repression of $P_{RM}$ provides only a modulatory role in prophage induction. In their $\lambda lacF^{dim}$ phages, the $cro^-$ gene was replaced by the gene for a dimeric lac repressor, and lac operators were inserted at $P_L$ and $P_R$ to mimic Cro’s repression of the lytic promoters. The lac repressor was unable to directly repress $P_{RM}$ as no lac operator was inserted there. A larger UV dose was required to give half-maximal phage production from lysogens of the most wild-type-like of the variants, $\lambda AWCF$, compared with the parental phage, $\lambda JL351$. However, we believe that it is not possible to confidently attribute the induction defect in these $\lambda lacF^{dim}$ phages to a lack of repression of $P_{RM}$ by LacI because it is not clear that this is the only regulatory difference between the $\lambda lacF^{dim}$ phages and $\lambda JL351$. Specifically, the insertion of lac operators just downstream from the $P_L$ and $P_R$ promoters may alter the basal activities of these promoters, and their control by LacI is likely to differ from normal Cro regulation. Thus, the expression of the $\lambda$ lytic genes may well be different in the $\lambda lacF^{dim}$ phages and $\lambda JL351$. In addition, although Atsumi and Little showed that $P_{RM}$ in $\lambda AWCF$ is not directly repressed by lac repressor, their data indicated that the presence of lac repressor interferes with CI activation of $P_{RM}$. As CI and lac repressors are both present during prophage induction, this effect may partially substitute for Cro repression of $P_{RM}$. Also, the $\lambda lacF^{dim}$ phages and $\lambda JL351$ carry a mutation in the $cl$ gene that increases the sensitivity of the prophage to UV induction, probably acting by reducing lysogenic CI levels. This would most likely reduce the impact of the loss of Cro repression of $P_{RM}$ in comparison with wild-type $\lambda$. Thus, we believe that our results provide a more valid
measurement of the importance of Cro repression of P_{RM} in λ prophage induction.

**Cro repression of P_{RM} prevents recovery of CI**

Our results indicate that Cro’s action at P_{RM} is necessary primarily to prevent recovery of CI levels rather than affecting the rate at which CI levels fall after UV treatment. Up until ~20 min after a 10 J/m² UV treatment, we saw no difference in the induction of P_{R} activity in the O_{R}3- and O_{R}3-x3 CI–Cro switch reporters [Fig. 5B]. Also, direct measurements indicated that CI fell to similar levels in the two strains by 20 min [Fig. 5C]. Although there is some P_{R} activity in the early stages of induction, we imagine that this produces insufficient Cro to affect P_{RM} significantly. Even if Cro does begin to repress P_{RM} before 20 min, there will be a delay before this repression impacts on CI production because of the time required for the existing CI mRNA to degrade. However, as Cro accumulates and RecA activity wanes due to repair of the UV damage, Cro’s ability to repress P_{RM} would have a more significant impact on CI levels. Accordingly, the P_{R} activity and CI levels in the O_{R}3- and O_{R}3-x3 CI–Cro switch reporters diverge 20 min after UV. In the O_{R}3- strain, a low CI level and a lack of CI repression of P_{R} were maintained throughout the vast majority of cells until ~240 min after UV. In contrast, a gradual increase in repression of P_{R} was apparent after 20 min in the induction of the O_{R}3-x3 CI–Cro switch, with re-establishment of P_{R} repression and lysogenic CI levels complete by 80–120 min. Although the timing of this re-establishment of CI in the switch reporter seems slow in the context of the ~50 min required for phage production after UV, we expect that CI recovery would occur more quickly after UV induction of a prophage due to replication of the λ genome and consequent increased number of CI genes.

The whole-phage studies also support the idea that the main function of Cro repression of P_{RM} is to prevent recovery of CI levels after prophage induction. The defect in P_{RM} repression by Cro strongly reduced the number of phages produced per induced cell (threefold, eightfold, and 22-fold reductions; λ.WT vs. O_{R}3-x3 at 10 J/m² UV and at 5 J/m² UV; and λ.O_{R}3-rI vs. O_{R}3-KO at 10 J/m², respectively) [Table 1] but had less of an effect on the fraction of cells induced [1.3-fold, twofold, and threefold reductions for the same comparisons] (Table 1). Thus, Cro repression of P_{RM} does not so much affect the probability of an irreversible commitment to lytic development, but more affects the execution of lytic development subsequent to that commitment. The recovery of CI levels seen in the absence of Cro repression of P_{RM} therefore appears to have a significant inhibitory effect on the efficiency of lytic development. In fact, the reduction in the fraction of induced cells in the O_{R}3-x3 and O_{R}3-KO mutants may in part be due to CI recovery being able to block lytic development completely in some cells.

These results contradict the view that the UV dose and Cro repression of P_{RM} only affect the probability of crossing some decisive induction threshold and do not affect the execution of lytic development after that decision ([Atsumi and Little 2006]). If this view were correct, then one would expect the UV dose and Cro repression of P_{RM} to have large effects on the fraction of cells induced and small effects on the number of phage produced per induced lysogen, whereas we saw the reverse. Even λ.WT lysogens had a twofold reduced number of phages produced per induced cell at 5 J/m² compared with 10 J/m² [Table 1], showing that even when Cro repression of P_{RM} is intact, there can be substantial inhibition of lytic development after induction. Thus, Cro repression of P_{RM} significantly improves the execution of lytic development after prophage induction but does not make it perfect.

**Concluding remarks**

A stable immune state of the CI–Cro regulatory circuit is necessary for stable lysogeny, but a stable anti-immune state is clearly not necessary for λ lytic development itself. Instead, the function of the bistability that results from Cro’s strong repression of P_{RM} is to improve the transition out of lysogeny into lytic development. We imagine that the selective advantage of more efficient prophage induction would easily be sufficient to drive the evolution of a fully bistable system from a monostable one, in which Cro repression of P_{RM} was weak or absent. Studies of other temperate phages support the idea that λ’s stable anti-immune state is not just an accident of evolution. To our knowledge, all temperate phages, including many unrelated to λ, have some kind of anti-immunity factor, often a small Cro-like DNA-binding protein, and it has been reported in many cases that the immunity/anti-immunity regulatory circuit of these phages is bistable. Thus, it seems that there is selective pressure to make the anti-immunity regulation strong enough to preclude the immune state. We expect that this bistability serves similar functions in these phages as in λ but that the impact on prophage induction and on lytic development after infection may vary, depending on features such as the mechanism of induction, the length of the lytic cycle and the regulation of the immunity factor.

By its repression of the promoters at O_{R} and O_{L}, Cro antagonizes the production of both major anti-lytic regulators, CI and CII. Cro repression of P_{R} (and P_{I}) is needed after infection to prevent CII overexpression during lytic development. As we have shown, during lytic development after prophage induction, Cro repression of P_{RM} is important to prevent CI resurgence. It seems likely that Cro’s anti-CII action is also important after prophage induction, although this has not been tested directly. It would be interesting to see whether the defective prophage induction of λ.O_{R}3-x3 is worsened by a cro mutant and in a cII-dependent manner.

The results presented here and previously [Dodd et al. 2001, 2004] show that O_{R}3, rather than being the “third wheel” on the O_{R} cycle, plays dual critical roles in λ’s transition from lysogeny to lytic development. First, O_{R}3 enables CI to repress P_{RM} and thus maintain a ly-
sogenic level of CI that is low enough to be effectively removed by RecA upon induction of the SOS system. Second, Op3 allows Cro to efficiently repress $P_{RM}$ and prevent the recovery of CI levels that would otherwise impede or even halt lytic development. The response of the Op3-KO mutant to a UV dose that induces every $\lambda WT$ lysogen (10 J/m²) (Table 1) shows that these activities of Op3 combine to provide a 500-fold improvement in phage production.

Materials and methods

Strains and mutations

E4300 [ = NK7049 [ΔlacIZYA·X74 galO·P308 StrR·Su·+] ] (Simons et al. 1987) was used for reporter assays, and C600 was used for phage experiments. Mutations were introduced by PCR-based methods. The Op2, Op3, and Pm mutations are shown in Figure 2A. The croΔ21 mutation removes positions 38109–38129 + Cro (G243–E30). The croTH mutation changes codons 26–28 TATCAAAAGC (YGS) to TACGAACGC (YER). The cl857 change is 37742C→T. The $P_{R_{KM}}$ mutation is a change of the –10 AAGTAT→GCGATGC. All lysogens were confirmed as single copy (Powell et al. 1994).

Phage constructions

Mutations in the cl–Oa–cro–cll region to be transferred to $\lambda$ were constructed in pPAP31 and crossed onto $\lambda$ b::kan.imm434 by in vivo recombination, as described previously for $\lambda$ imm434 (Dodd et al. 2001). b::kan.imm434 was obtained by crossing the b::kan insertion from pWL464 onto b::kan.imm434 (Supplemental Material, Michalowski et al. 2004). $\lambda$ b::kan is referred to as $\lambda WT$, and all Op mutants phage carry the b::kan insertion.

Reporter strains and assays

The lacZ reporters strains used in Figures 1–3 were constructed by cloning $\lambda$ DNA into pTL61T (Linn and St. Pierre 1990) for transcriptional fusions, or pRS414 (Simons et al. 1987) for translational fusions, recombining these fusions onto $\lambda$ RS45 YAOL or $\lambda$ RS45 YA and making single-copy lysogenes of these phages in E4300, as described previously (Dodd et al. 2001). Details are given in the Supplemental Material.

Cro or CI were expressed in the reporter strains from plac on plasmids pZEl5cro or pZC320cl (Dodd et al. 2001), controlled by lac repressor from pUHA1 (Lutz and Bujard 1997) and IPTG. Cells were cultured in LB (+antibiotics) and assayed using a kinetic microtiter plate-based LacZ assay (Dodd et al. 2001).

To make the CI–Cro switch reporters, $\lambda$ sequence extending from the P1 leader to within the cll gene was inserted into the plasmid vector pIT-SLlacZ, which was then integrated at $\lambda$attB in the chromosome of E4300. pIT-SLlacZ is based on the CRIM-integrating plasmid system (see Supplemental Material, Haldimann and Wanner 2001). The cl857, croTH+, and Op3-x3 alleles were introduced by reselection.

Western blotting

Single C600 lysogens were isolated and the level of CI protein in the strains was quantitated by Western blotting as described previously (Dodd et al. 2001), except that cells from –1 mL of log phase culture [final volumes were adjusted to give a constant OD₆₀₀] were disrupted with 40 μL of B-Per reagent [Pierce] containing 0.25 U/μL Benzonase [Novagen] and 0.2 μg/μL lysozyme [Sigma]. Detection was with a Cy5-labeled goat anti-rabbit secondary antibody (GE Healthcare), and blots were quantitated with a GE Typhoon imager.

Single step burst curves and frequency of lysogeny assays

For infection of C600 hosts, log phase cultures in LBM (LB, 10 mM MgSO₄) plus 0.2% maltose were concentrated ~10-fold by centrifugation at 37°C and infected with prewarmed phage at a multiplicity of addition <0.01 for 10 min at 37°C (adsorption was 95%–99% efficient). The infection was diluted at least ~100-fold in LBM and incubated with shaking at 37°C.

To follow the phage burst, aliquots were removed at various times and immediately diluted and plated for infectious centers [IC] with C600 indicator bacteria [in LB] and 3 mL of molten top agar [0.7% agar, 10 mM MgSO₄] on TB plates (1% Bacto-tryptone, 0.5% NaCl, 1.5% Bacto-agar [Difco]).

To measure the frequency of lysogeny, an aliquot taken 15 min after initial infection was immediately diluted and plated on C600 indicator for IC or for free phage [FP, treated with chloroform to kill bacteria before being plated with indicator]. After 25 min, the infection was plated with 3 mL of molten top agar onto LB plates containing 20 μg/mL kanamycin, to measure the number of lysogens formed. Total phage added was experimentally equated to the sum of the number of IC and lysogens. The total number of infections was the total phage added minus the FP. The frequency of lysogeny was the [number of lysogens divided by the total number of infections] × 100.

UV and temperature induction of prophage

For UV induction, log phase cultures of C600 lysogens in LB [at cell density of ~1 x 10⁸ colony-forming units [cfu/mL]] were harvested by centrifugation at 4°C, resuspended neat in M9 salts [Miller 1972] and 5 mL [+1 μL of 10% Tween20 for wetting] placed in a sterile 8.5-cm plastic Petri dish. Prior to UV exposure, an aliquot was plated on LB plates to assay cell density. After irradiation with a germicidal UV lamp under yellow lighting at 37°C, the culture was diluted ~1/5 into LB and incubated at 37°C with shaking. At a number of time points, aliquots were diluted and plated with indicator for IC and/or FP.

Untreated $\lambda$ lysogens plated with indicator bacteria were found to give plaques [very small in size] at a rate of ~6%–15%, depending on the mutant. Therefore, prior to UV treatment the culture was diluted and plated with indicator to measure the level of uninduced lysogens giving plaques [plaque-forming lysogens]. The number of induced lysogens was the preburst IC minus the number of plaque-forming lysogens.

The fraction of cells induced was the number of induced lysogens as a fraction of the lysogens present. The number of phage produced per lysogen was the FP post-burst divided by the number of induced lysogens.

Spontaneous induction was measured by plating dilutions of log phase cultures of C600 lysogens onto TB plates for colony-forming units, and assaying the culture supernatants [following centrifugation at 4°C for FP].

For the experiments shown in Figure 4D, log phase cultures of clts lysogens in LB at 30°C were temperature-induced by diluting 1000-fold into LB prewarmed to 39°C and incubating at 39°C with shaking. At early time points, aliquots were diluted and plated immediately with indicator to measure preburst IC.

To follow the burst curve, aliquots taken at different times were treated with chloroform before being diluted and plated with indicator to measure the number of FP present.

UV induction of the $\lambda$ switch reporter

The $\lambda$ switch reporter strains were treated with UV as described above. After 1/3–1/5 dilution into LB, cultures were grown at
37°C with shaking and were diluted to maintain cells in log phase growth. Aliquots taken at various times were collected on ice and analyzed by LacZ assay and CI Western blotting.

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References
Adhya, S. and Gottesman, M. 1982. Promoter occlusion: Transcription through a promoter may inhibit its activity. Cell 29: 939–944.

Atsumi, S. and Little, J.W. 2006. Role of the lytic repressor in prophage induction of phage $\lambda$ as analyzed by a module-replacement approach. Proc. Natl. Acad. Sci. 103: 4558–4563.

Baek, K., Svenningsen, S., Eisen, H., Sneppen, K., and Brown, S. 2003. Single-cell analysis of $\lambda$ immunity regulation. J. Mol. Biol. 334: 363–372.

Bailone, A., Levine, A., and Devoret, R. 1979. Inactivation of prophage $\lambda$ repressor in vivo. J. Mol. Biol. 131: 553–572.

Court, D.L., Oppenheim, A.B., and Adhya, S.L. 2007. A new look at bacteriophage $\lambda$ genetic networks. J. Bacteriol. 189: 298–304.

Darling, P.J., Holt, J.M., and Ackers, G.K. 2000. Coupled energetics of a cro repressor self-assembly and site-specific DNA operator binding II: Cooperative interactions of cro dimers. J. Mol. Biol. 302: 625–638.

Dodd, I.B., Perkins, A.J., Tsemitsidis, D., and Egan, J.B. 2001. Octamerization of $\lambda$ CI repressor is needed for effective repression of $P_{lam}$ and efficient switching from lysogeny. Genes & Dev. 15: 3013–3022.

Dodd, I.B., Shearwin, K.E., Perkins, A.J., Burr, T., Hochschild, A., and Egan, J.B. 2004. Cooperativity in long-range gene regulation by the $\lambda$ CI repressor. Genes & Dev. 18: 344–354.

Eisen, H., Brachet, P., Pereira da Silva, L., and Jacob, F. 1970. Regulation of repressor expression in $\lambda$. Proc. Natl. Acad. Sci. 66: 855–862.

Folkmanis, A., Malzman, W., Mellon, P., Skalka, A., and Ephrath, H. 1977. The essential role of the cro gene in lytic development of bacteriophage $\lambda$. Virology 81: 352–362.

Haldimann, A. and Wanner, B.L. 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J. Bacteriol. 183: 6384–6393.

Herskovitz, I. and Hagen, D. 1980. The lysis-lysogeny decision of phage $\lambda$: Explicit programming and responsiveness. Annu. Rev. Genet. 14: 399–445.

Johnson, A.D. 1980. “Mechanism of action of the $\lambda$ cro protein.” Ph.D. thesis, Harvard University, Cambridge, MA.

Johnson, A., Meyer, B.J., and Ptashne, M. 1978. Mechanism of action of the cro protein of bacteriophage $\lambda$. Proc. Natl. Acad. Sci. 75: 1783–1787.

Johnson, A.D., Fottee, A.R., Lauer, G., Sauer, R.T., Ackers, G.K., and Ptashne, M. 1981. $\lambda$ Repressor and cro—Components of an efficient molecular switch. Nature 294: 217–223.

Kalonisis, B., Dodd, I.B., and Egan, J.B. 1986. Control of gene expression in the P2-related template coliphages. III. DNA sequence of the major control region of phage 186. J. Mol. Biol. 191: 199–209.

Kaufman, S.A. 1973. Control circuits for determination and transdetermination. Science 181: 310–318.

Kobiler, O., Rokney, A., Friedman, N., Court, D.L., Stavans, J., and Oppenheim, A.B. 2005. Quantitative kinetic analysis of the bacteriophage $\lambda$ genetic network. Proc. Natl. Acad. Sci. USA 102: 4470–4475.

Krinke, L., Mahoney, M., and Wulf, D.L. 1991. The role of the OOP antisense RNA in coliphage $\lambda$ development. Mol. Microbiol. 5: 1265–1272.

Lewin, B. 1999. Genes VII. Oxford University Press, New York.

Linn, T. and St. Pierre, R. 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of lacZ. J. Bacteriol. 172: 1077–1084.

Little, J.W. 1984. Autodigestion of lexA and phage $\lambda$ repressors. Proc. Natl. Acad. Sci. USA 81: 1375–1379.

Lutz, R. and Bujard, H. 1997. Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and ArcC/II-12 regulatory elements. Nucleic Acids Res. 25: 1203–1210.

Meyer, B.J., Maurer, R., and Ptashne, M. 1980. Gene regulation at the right operator [OR] of bacteriophage $\lambda$. II. OR1, OR2, and OR3: Their roles in mediating the effects of repressor and cro. J. Mol. Biol. 139: 163–194.

Michalowski, C.B., Short, M.D., and Little, J.W. 2004. Sequence tolerance of the phage $\lambda$ PRM promoter: Implications for evolution of gene regulatory circuitry. J. Bacteriol. 186: 7988–7999.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Murray, N.E. and Gann, A. 2007. What has phage $\lambda$ ever done for us? Curr. Biol. 17: R305–R312. doi: 10.1016/j.cub.2007.03.006.

Neubauer, Z. and Calef, E. 1970. Immunity phase-shift in defective lysogens: Non-mutational hereditary change of early regulation of $\lambda$ prophage. J. Mol. Biol. 51: 1–13.

Powell, B.S., Rivas, M.P., Court, D.L., Nakamura, Y., and Turnbough Jr., C.L. 1994. Rapid confirmation of single copy $\lambda$ prophage integration by PCR. Nucleic Acids Res. 22: 5765–5766.

Ptashne, M. 2004. A genetic witch: Phage $\lambda$ revisited. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarai, A. and Takeda, Y. 1989. $\lambda$ repressor recognizes the approximately twofold symmetric half-operator sequences asymetrically. Proc. Natl. Acad. Sci. USA 86: 6513–6517.

Simons, R.W., Houman, F., and Kleckner, N. 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene 53: 85–96.

Svenningsen, S.L., Costantino, N., Court, D.L., and Adhya, S. 2005. On the role of Cro in $\lambda$ prophage induction. Proc. Natl. Acad. Sci. 102: 4465–4469.

Takeda, Y. 1979. Specific repression of in vitro transcription by the Cro repressor of bacteriophage $\lambda$. J. Mol. Biol. 127: 177–189.

Takeda, Y., Sarai, A., and Rivera, V.M. 1989. Analysis of the sequence-specific interactions between Cro repressor and operator DNA by systematic base substitution experiments. Proc. Natl. Acad. Sci. USA 86: 439–443.

Toman, Z., Dambly-Chaudiere, C., Tenenbaum, L., and Radman, M. 1985. A system for detection of genetic and epigenetic alterations in Escherichia coli induced by DNA-damaging agents. J. Mol. Biol. 186: 97–105.