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 P_{RM} promoter for CI has remained unclear. To address this, we created binding site mutations that strongly impaired Cro repression of P_{RM} with only minimal effects on CI regulation of P_{RM} . 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 lysogens, repression of P_{RM} 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.

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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 P_{RM} and binds cooperatively to two operators, O_R1 and O_R2 , to repress the P_R lytic promoter and thus block transcription of the *cro* gene. CI simultaneously activates transcription of its own gene from P_{RM} . In the anti-immune (Cro-dominant) state, Cro is expressed from P_R and prevents CI expression, presumably by virtue of its high affinity for O_R3 , where it can bind to repress P_{RM} (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 the anti-immune state of the switch and the repression of P_{RM} 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 P_{RM} , that is critical for lytic development after infection. Cro binds with highest affinity to O_R3 but it also binds to O_R1 and O_R2 at P_R and to the operators at P_L (Johnson et al. 1978; Takeda 1979; Darling et al. 2000), from where it can exert at least a two- to four-fold 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*, *cIII*, *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 P_{RM} , but instead results from

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Figure 1. Cro repression of P_{RM} . (A) Regulation of lytic and lysogenic promoters by Cro. (B) Repression of P_{RM} by single-copy *cro* in *cis*. The chromosomally integrated P_{RM} -*lacZ* transcriptional reporter carries the O_R -*cro* region inserted so that *lacZ* is transcribed from P_{RM} but is translated from the wild-type *lacZ* ribosome-binding site (RBS). Wild-type Cro, or a mutant protein carrying a deletion within its HTH DNA-binding motif (*cro* Δ 21), is expressed in *cis* from P_R . (C) Repression of CI expression by single-copy *cro* in *cis*. The P_{RM} -*cI* translational reporter is as in B, except that codon 20 of *cI* is fused to codon 10 of *lacZ*, such that *lacZ* is transcribed from P_{RM} and translated from the *cI* RBS. Errors are 95% confidence intervals.

overexpression of the CII and CIII proteins, as it can be suppressed by *cII*⁻ and *cIII*⁻ mutations (Folkmanis et al. 1977). CII, stabilized by CIII, activates an alternative promoter for *cI*, P_{RE} . CII also activates the P_I promoter for the integrase protein that inserts the λ genome as a prophage into the host chromosome, and the P_{AQ} promoter that inhibits the production of the late gene activator Q (Kobiler 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 O_R 3 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 O_R 3, which results in a threefold increase in lysogenic CI levels that is inhibitory to prophage induction (Dodd et al. 2001). Although Svenningsson 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 *cI*-*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 multi-copy 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 *cl* 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-r1} 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_R1 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_{R2-KO} mutant (Fig. 2A). It was only when the O_{R2-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. 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 ΔG 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 ΔG 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 (*cro* $\Delta c21$ 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_{R2-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 Δcro 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_R2 and O_R3 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_R3 -x3 mutant combines three mutations at O_R3 (Fig. 2A) and, like the O_R3 -KO mutant, gave only weak repression of P_{RM} by Cro in *cis*. The addition of the O_R2 -r204 change to the O_R3 -x3 mutant, to give the $O_R2/3$ -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_R3 -x3 mutation had small effects on regulation of P_{RM} by CI. Although the $O_R2/3$ -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_R3 -KO mutation further. We expected this mutation to strongly reduce CI repression of P_{RM} and therefore included the O_R3 -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_R3 -x3 mutation slightly increased the intrinsic activity of P_{RM} (~1.3-fold), while the $O_R2/3$ -x4 mutation increased it somewhat more (~1.7-fold). Surprisingly, the O_R3 -KO mutation did not change basal P_{RM} activity.

Regulation of P_{RM} by CI Figure 3A shows the response of P_{RM} -*lacZ* reporters carrying the O_R mutations to a range of CI levels supplied by an isopropyl thio-β-D-galactoside (IPTG)-controlled expression plasmid. The wild-type (WT) and O_R3 -x3 mutant showed quite similar profiles: activation at low CI concentrations due to CI binding at O_R2 , followed by repression at higher CI concentrations as O_R3 becomes occupied. CI repression of P_{RM} in the O_R3 -x3 mutant was slightly stronger, suggesting that CI may bind slightly better to O_R3 -x3. Despite its higher basal activity, P_{RM} in the $O_R2/3$ -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_R3 -KO mutation showed the same response of P_{RM} to CI previously described for the O_R3 -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 -*lacZ* reporters, carrying the O_R mutations, to Cro or CI supplied from plas-

Figure 3. Effects of the mutations on other aspects of O_R regulation. Response of P_{RM} -*lacZ* reporters (A) and P_R -*lacZ* reporters (B,C) to CI (A,C), or Cro (B) supplied by IPTG-inducible plasmids. The λ fragment extends from the +62 of P_{RM} to the +43 of P_R . The inset in C shows reporter expression with CI supplied from a λ att80 prophage. (D) Translational fusion reporters showing the effect of the O_R mutations on *cI* translation from P_{RE} mRNA. λ P_{RE} is substituted by the constitutive 186 *pB* promoter. (A–D) Error bars and ranges are 95% confidence limits.

mids or to CI supplied by a λ prophage. Although there are some small reproducible effects of the O_R3 and O_R2 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 $O_R2/3$ -x4 mutant (Fig. 3C), consistent with weakening of Cro binding to O_R2 (Meyer et al. 1980). Inciden-

tally, this is possibly the first demonstration that Cro binding to O_R3 does not affect P_{RM} .

Translation of cI from P_{RE} mRNA The O_R mutations lie just upstream of the cI start codon, and we realized that they could well affect ribosome binding for cI translation from the P_{RE} mRNA (Fig. 2A). The cI mRNA produced from P_{RM} begins at the cI start codon and therefore is not affected by the O_R 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 P_{RE} promoter was substituted by the constitutive pB promoter from bacteriophage 186 (Kalionis et al. 1986). The cro gene and P_R 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 P_{RM} transcript (see Fig. 1C). The O_R3 -x3, O_R3 -r1, and $O_R2/3$ -x4 mutations had relatively small effects on cI translation from the P_{RE} mRNA, but the O_R3 -KO mutation increased this translation sixfold. Although this drastic effect would seem to render the O_R3 -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 O_R3 and O_R2 mutants provided two pairs of mutants whose comparison was expected to be instructive with regard to the role of Cro repression of P_{RM} . The major difference between O_R -WT and O_R3 -x3 is that the mutant retains only ~12% of P_{RM} repression by single-copy Cro (1.5/11.9). However, the O_R3 -x3 mutation increases basal P_{RM} by ~1.3-fold and slightly increases CI repression of P_{RM} . We included the $O_R2/3$ -x4 mutant as an adjunct to this pair because of its greater defect in Cro repression of P_{RM} . However, $O_R2/3$ -x4 has a larger effect on basal P_{RM} activity (up 1.7-fold) and reduces CI-stimulated P_{RM} activity by ~25%.

The other pair of mutants, O_R3 -r1 and O_R3 -KO, differ in that O_R3 -KO retains only 15% of Cro repression of P_{RM} (1.8/11.9), while Cro repression of P_{RM} in O_R3 -r1 is normal. A caveat to the comparison of this pair is that translation of cI from the P_{RE} mRNA is approximately ninefold more efficient in λ . O_R3 -KO compared with λ . O_R3 -r1 (340/38) (Fig. 3D).

The four O_R 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 O_R 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 O_R mutations had only small effects on phage production after infection, confirming that Cro repression of

P_{RM} 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 O_R3 -x3 mutant was slightly lower than wild type (75%) (Table 1). Similarly, the O_R3 -KO mutant burst size was 66% of λ . O_R3 -r1. The burst deficit did not increase with the further loss of Cro repression of P_{RM} in the $O_R2/3$ -x4 mutant.

The proportion of infected cells that formed lysogens was slightly increased in the mutants with defective Cro repression of P_{RM} . The frequency of lysogeny of the O_R3 -x3 mutant was 30% higher than the wild type and was 25% higher for λ . O_R3 -KO compared with λ . O_R3 -r1 (Table 1). A larger increase in lysogenization was seen with λ . $O_R2/3$ -x4.

These results are consistent with Cro repression of P_{RM} causing a slight enhancement of lytic development and a slight decrease in lysogenization after infection, but alternative explanations are possible. In particular, for the O_R3 -KO mutant, these effects could be due to the large increase in the efficiency of CI production from P_{RE} 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 P_I , P_{AQ} 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 P_{RM} in the lysogen, altered CI regulation of P_{RM} 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 O_R3 -KO mutation or the O_R3 -r1 mutation caused a approximately threefold increase in lysogenic CI level, as expected due to lack of CI negative autoregulation. The O_R3 -x3 and the $O_R2/3$ -x4 mutant lysogens contained 72% and 57% of the wild-type CI level, respectively, consistent with their altered regulation of P_{RM} by CI (Fig. 3A). On the basis of these measurements alone, one would expect the O_R3 -x3 and the $O_R2/3$ -x4 mutant lysogens to be somewhat more easily induced than wild type and the O_R3 -KO mutant lysogen to be induced poorly, like the O_R3 -r1 mutant (Dodd et al. 2001).

The O_R 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 O_R 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 O_R mutant phages. (A) Phage production after infection. C600 cells were infected at a phage:bacterium ratio <0.01 with λ WT or O_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 λ WT or O_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 ($n = 8$ or 9). (C) Phage production after UV irradiation (10 J/m^2) of C600 lysogens. (D) Temperature induction of $\lambda.cIts$ and $\lambda.cIts.O_R3-x3$ prophages. C600 lysogens were transferred from 30°C to 39°C and samples were assayed for FPs. (E) UV induction (as in C) of $\lambda.O_R3-r1$ and $\lambda.O_R3-KO$ prophages and their P_{RE}^- derivatives. For the O_R3-r1 P_{RE}^+ and O_R3-r1 P_{RE}^- phages, respectively, 56% and 50% of lysogens were induced, producing 26 and 32 phage per irradiated lysogen. For the O_R3-KO P_{RE}^+ and O_R3-KO P_{RE}^- phages, respectively, 18% and 30% of lysogens were induced, producing on average 0.6 and 0.9 phage per irradiated lysogen.

lowing calculation of the average number of phage produced per induced lysogen. After a UV dose of 10 J/m^2 , which was sufficient to induce all of the λ .WT lysogens, the $\lambda.O_R3-x3$ and $\lambda.O_R2/3-x4$ lysogens produced only about one-fifth of the number of phage produced by the λ .WT lysogen (Table 1). At the suboptimal dose of 5 J/m^2 , which induced only 75% of the λ .WT lysogens, the defect for $\lambda.O_R3-x3$ was even more severe, with ~ 16 -fold fewer phage produced than wild type. The defect in the $\lambda.O_R3-x3$ mutant at each dose was in part because a smaller fraction of lysogens was induced but was mainly due to fewer phages being produced in those cells that were induced. The defect in the $\lambda.O_R2/3-x4$ lysogen was almost entirely due to a lowered burst size.

Cultures of $recA^+$ λ lysogens contain low levels of phage even in the absence of added inducing stimuli, due to rare SOS-dependent transitions to lytic development. The relative numbers of phages per cell in cultures of different lysogenic strains is a measure of the relative rates of phage production (assuming the same rate of phage loss; e.g., by absorption). The O_R3-x3 and $O_R2/3-x4$ mutants showed an approximately fivefold lower spontaneous phage production than wild type (Table 1), similar to the 10-J/m^2 UV defect.

As expected from its high lysogenic CI concentration, the $\lambda.O_R3-r1$ lysogen was poorly induced by UV, with only half the cells producing phage at 10 J/m^2 . The defect in the O_R3-KO mutant was much more severe, with phage production reduced 50-fold below that of $\lambda.O_R3-r1$ (Table 1). As seen for the O_R3-x3 and $O_R2/3-x4$ mutants, it was the low number of phages produced per induced cell that was the prime cause of the $\lambda.O_R3-KO$ defect. Spontaneous phage production was also reduced, though less severely, for $\lambda.O_R3-KO$ compared with $\lambda.O_R3-r1$ (sevenfold).

If the prophage induction defect of $\lambda.O_R3-x3$ relative to λ .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 cI^+ gene of λ .WT and $\lambda.O_R3-x3$ with the temperature-sensitive cI_{857} allele. Figure 4D shows that after induction of the $cIts$ lysogens by raising the temperature to 39°C , there was very little difference in phage production for the O_R3-x3 and wild-type lysogens (wild type and O_R3-x3 gave 259 ± 71 and 231 ± 33 phage per induced cell, respectively; $\pm$ SD, $n = 2$). Clearly, the prophage induction defect caused by the O_R3-x3 mutation is dependent on the presence of active CI.

We expected that the poor prophage induction of the O_R3-KO mutant relative to the O_R3-r1 mutant was caused by increased CI production due to lack of Cro repression of P_{RM} in the O_R3-KO mutant. However, the increased translation efficiency of cI from the P_{RE} mRNA in the O_R3-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 phage production from a lysogen (Baek 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 O_R3-KO mutant. We found that introduction of a P_{RE}^- mutation into the O_R3-r1 and O_R3-KO prophages had very little effect on phage production after a 10 J/m^2 UV dose, with the O_R3-KO P_{RE}^- mutant retaining the induction defect relative to O_R3-r1 P_{RE}^- (Fig. 4E). Thus the defective prophage induction seen for the O_R3-KO mutant is not due to increased CI expression from P_{RE} mRNA.

Table 1. Behavior of the mutant phages

Phage property	λWT	λO_{R3-x3}	$\lambda O_{R2/3-x4}$	λO_{R3-r1}	λO_{R3-KO}
<u>Phage production after infection</u>					
Burst size ^a	285 ± 44 (10)	204 ± 42 (3)	207 ± 49 (3)	270 ± 32 (3)	165 ± 27 (4)
% Wild type ^b	100	75 (66–86)	76 (54–107)	89 (74–108)	59 (47–72)
% O_{R3-r1}	—	—	—	100	66
<u>Establishment of lysogeny</u>					
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	100	130 (120–150)	220 (180–270)	120 (100–140)	150 (120–180)
% O_{R3-r1}	—	—	—	100	125
<u>UV induction of prophage: 10 J/m²</u>					
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	32 (21–48)
Phage per lysogen	255 ± 137	52 ± 32	55 ± 19	36 ± 26	0.7 ± 0.6
% Wild type	100	22 (14–34)	22 (12–40)	13 (9–18)	0.2 (0.1–0.4)
% O_{R3-r1}	—	—	—	100	1.8 (1.1–2.9)
Phage per induced cell ^c	231 ± 120	69 ± 41	54 ± 9	66 ± 40	2.9 ± 1.4
% Wild type	100	32 (17–61)	24 (12–49)	27 (20–38)	1.3 (1.2–1.4)
% O_{R3-r1}	—	—	—	100	4.6 [3.3–6.6]
<u>UV induction of prophage: 5 J/m²</u>					
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	33 ± 7		
% Wild type	100	6 (3–12)	36 (25–53)		
Phage per induced cell	117 ± 23	15 ± 19	46 ± 12		
% Wild type	100	12 (5–29)	39 (32–46)		
<u>Spontaneous phage production from lysogen</u>					
Phage per lysogen	44 × 10 ⁻⁵ (6)	8.3 × 10 ⁻⁵ (6)	7.7 × 10 ⁻⁵ (5)	15 × 10 ⁻⁵ (10)	2.4 × 10 ⁻⁵ (7)
% Wild type	100	19 (11–33)	22 (14–35)	35 (25–50)	5 (4–8)
% O_{R3-r1}	—	—	—	100	14

^aBurst 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.

^bWhen a range of percentages are given in parentheses, the values in bold are averages of the ratios for λWT or λO_{R3-r1} obtained within each experiment, and the ranges indicate the 95% confidence limits calculated using the logarithms of these ratios.

^cValues 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 λ genes. We chose the O_{R3-x3} mutation as the best comparison with wild-type.

We measured P_R transcription using chromosomal *cIts*. P_{RM} , P_R , *cro*, *lacZ* 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 CIs 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 persis-

tent 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 *cIt+* versions of the above reporters. Figure 5B shows a time course of LacZ production from P_R after a 10 J/m² 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 *O_R3-x3* mutation on UV induction of the CI-Cro switch. (A) Structure of the chromosomally integrated *cI.P_{RM}.P_R.cro.lacZ* reporter constructs. LacZ activity from *P_R* (B), and Western blotting of CI (C) following 10 J/m² UV treatment of the *cro⁺* reporters. (D) Relative LacZ activities in the *O_R3⁺* and *O_R3-x3* reporters after different UV doses. The strains carrying the *croHTH⁻* mutation showed that the *O_R3-x3* mutation made no difference to *P_R* LacZ activity in the absence of Cro (open symbols, 10 J/m² UV treatment).

represses *P_R* but also to the approach of LacZ accumulation to steady state, where the rate of LacZ production from *P_R* 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 *P_R* reporter is expected to be ~180–220 U, reduced from the ~1150 U seen for *P_R* 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 termination at *t_{R1}* (approximately threefold reduction) (Fig. 5A; A. Palmer, unpubl.). Thus, *P_R* remained highly active in most cells of the *O_R3⁺cro⁺* strain for the duration of the experiment. In the *O_R3-x3 cro⁺* reporter, the increase in LacZ units slowed in comparison with *O_R3⁺cro⁺* soon after 40 min and LacZ accumulation halted after 80 min, indicating that *P_R* 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 *P_R* is now strongly repressed and no new LacZ protein is being made. These results show that in the absence of Cro repression of *P_{RM}*, CI activity becomes re-established after UV induction, reducing and eventually fully repressing *P_R* activity.

The magnitude of the improvement in *P_R* activity due to Cro repression of *P_{RM}* depends on the UV dose. Figure 5D shows the relative LacZ levels produced from *P_R* in the presence compared with the absence of Cro repression of *P_{RM}* at three UV doses. At the high dose of 20 J/m², Cro repression of *P_{RM}* has relatively little effect on *P_R* expression over the time of the experiment, presumably because RecA activity persists sufficiently to inactivate any CI made from *P_{RM}*. At 5 J/m², the Cro/*P_{RM}* 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 *P_{RM}* unless the promoter is repressed by Cro.

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

Figure 6. Repression of *P_{RM}* by single-copy *cro* in *cis* in the presence of CI. The *cro⁺* curve was obtained using the *P_{RM}.lacZ* transcriptional reporter in Figure 1B. The reporter for the Δ *cro* curve is the same except that the *cro* gene is truncated at the +43 position of *P_R*. CI was supplied with an IPTG-inducible expression plasmid system, with 150 μM IPTG producing a *P_{RM}* 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 *P_R.lacZ* (Δ *cro*) reporter to CI (LacZ values divided by 5) (Dodd et al. 2004).

onstrate 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 λ phage. The O_R3 -x3 mutation caused an ~25% decrease in burst size and an ~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_R3 -KO mutation compared with the O_R3 -r1 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_R3 -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_R3 -r1 and O_R3 -KO mutants, the burst size and lysogenization changes in the λ . O_R3 -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_R3 -x3 and O_R3 -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/m²) that induced all λ .WT lysogens, phage production from λ . O_R3 -x3 lysogens was almost fivefold lower than wild type. Phage production from λ . O_R3 -x3 lysogens was 16-fold lower than wild type at the lower dose of 5 J/m², which induced 75% of λ .WT lysogens. At the 10 J/m² dose, 50% of λ . O_R3 -r1 lysogens were induced and phage production from the λ . O_R3 -KO lysogens was 55-fold lower than for λ . O_R3 -r1. These effects are much stronger than can be explained by the other effects of the mutations. The O_R3 -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_R3 -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 λ . O_R3 -KO and λ . O_R3 -r1 is the increased *cI* translation from P_{RE} mRNA in the O_R3 -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 *cI*₈₅₇. P_{RM} . P_R .*cro*.*lacZ* reporters did not increase P_R activity at temperatures that partially released P_R from repression by the temperature-sensitive mutant CI₈₅₇ 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 λ prophage induction.

Atsumi and Little (2006) concluded that Cro repression of P_{RM} provides only a modulatory role in prophage induction. In their λ *lacI*^{dim} phages, the *cro* gene was replaced by the gene for a dimeric *lac* repressor, and *lac* operators were inserted at P_R and P_L 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, λ AWCF, compared with the parental phage, λ JL351. However, we believe that it is not possible to confidently attribute the induction defect in these λ *lacI*^{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 λ *lacI*^{dim} phages and λ 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 λ lytic genes may well be different in the λ *lacI*^{dim} phages and λ JL351. In addition, although Atsumi and Little showed that P_{RM} in λ 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 λ *lacI*^{dim} phages and λ JL351 carry a mutation in the *cI* 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 λ . 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_R3^+ and $O_R3\text{-}x3$ 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_R3^+ and $O_R3\text{-}x3$ CI–Cro switch reporters diverge 20 min after UV. In the O_R3^+ strain, a low CI level and a lack of CI repression of P_R were maintained in at least the vast majority of cells up 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 $O_R3\text{-}x3$ 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; $\lambda.WT$ vs. $O_R3\text{-}x3$ at 10 J/m² UV and at 5 J/m² UV, and $\lambda.O_R3\text{-}r1$ vs. $O_R3\text{-}KO$ 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 $O_R3\text{-}x3$ and $O_R3\text{-}KO$ 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 $\lambda.WT$ 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_L) 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 $\lambda.O_R3\text{-}x3$ is worsened by a *cro*[−] mutation and in a *cII*-dependent manner.

The results presented here and previously (Dodd et al. 2001, 2004) show that O_R3 , rather than being the “third wheel” on the O_R bicycle, plays dual critical roles in λ 's transition from lysogeny to lytic development. First, O_R3 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, O_R3 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 O_R3 -KO mutant to a UV dose that induces every λ .WT lysogen (10 J/m^2) (Table 1) shows that these activities of O_R3 combine to provide a 500-fold improvement in phage production.

Materials and methods

Strains and mutations

E4300 [= NK7049 ($\Delta lacIZYA$)X74 galOP308 Str^R 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 O_R2 , O_R3 , and P_R^- mutations are shown in Figure 2A. The *cro* $\Delta 21$ mutation removes positions 38109–38129 = Cro(G24-I30) Δ . The *cro*HTH⁻ mutation changes codons 26–28 TATCAAAGC (YGS) to TACGAACGC (YER). The *cl*₈₅₇ change is 37742C → T. The P_{RE}^- mutation is a change of the –10 AAGTAT → GCATGC. All lysogens were confirmed as single copy (Powell et al. 1994).

Phage constructions

Mutations in the *cl*- O_R -*cro*-*cII* region to be transferred to λ were constructed in pAP831 and crossed onto λ .*b::kan.imm434* by in vivo recombination, as described previously for λ .*imm434* (Dodd et al. 2001). λ .*b::kan.imm434* was obtained by crossing the *b::kan* insertion from pJWL464 onto λ .*imm434* (Supplemental Material; Michalowski et al. 2004). λ .*b::kan* is referred to as λ WT, and all O_R mutant phage carry the *b::kan* insertion.

Reporter strains and assays

The *lacZ* reporters strains used in Figures 1–3 were constructed by cloning λ DNA into pTL61T (Linn and St. Pierre 1990) for transcriptional fusions, or pRS414 (Simons et al. 1987) for translational fusions, recombining these fusions onto λ RS45 Δ YAOL or λ RS45 Δ YA and making single-copy lysogens 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 pZE15cro 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, λ sequence extending from the P_L leader to within the *cII* gene was inserted into the plasmid vector pIT-SLlacZY, which was then integrated at $\lambda attB$ in the chromosome of E4300. pIT-SLlacZY is based on the CRIM-integrating plasmid system (see Supplemental Material; Haldimann and Wanner 2001). The *cl*₈₅₇, *cro*HTH⁻, and O_R3 -x3 alleles were introduced by resection.

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 μ L of B-Per reagent (Pierce) containing 0.25 U/ μ L Benzonase (Novagen) and 0.2 μ g/ μ L 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 LBM) 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 μ g/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) $\times$ 100.

UV and temperature induction of prophage

For UV induction, log phase cultures of C600 lysogens in LB (at cell density of $\sim 1 \times 10^8$ colony-forming units [cfu]/mL) were harvested by centrifugation at 4°C, resuspended neat in M9 salts (Miller 1972) and 5 mL (+1 μ L 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 λ 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 lysogens present. The number of phage per induced cell was the average 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 *cIts* 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 λ switch reporter

The λ 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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