Cooperativity in long-range gene regulation by the λ CI repressor

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Effective repression of *cI* transcription from P_{RM} by the bacteriophage λ CI repressor requires binding sites (O_L) located 2.4 kb from the promoter. A CI tetramer bound to $O_L1.O_L2$ interacts with a tetramer bound near P_{RM} ($O_R1.O_R2$), looping the intervening DNA. We previously proposed that in this CI octamer:DNA complex, the distant O_L3 operator and the weak O_R3 operator overlapping P_{RM} are juxtaposed so that a CI dimer at O_L3 can cooperate with a CI dimer binding to O_R3. Here we show that O_L3 is necessary for effective repression of P_{RM} and that the repressor at O_L3 appears to interact specifically with the repressor at O_R3. The O_L3-CI-O_R3 interaction involves the same CI interface used for short-range dimer–dimer interactions and does not occur without the other four operators. The long-range interactions were incorporated into a physicochemical model, allowing estimation of the long-range interaction energies and showing the lysogenic state to be ideally poised for CI negative autoregulation. The results establish the λ system as a powerful tool for examining long-range gene regulatory interactions in vivo.

[*Keywords:* DNA looping; protein–protein interactions; multimerization; transcriptional control; lysogeny]

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In complex organisms, activation or repression of promoter activity by proteins bound to enhancer or silencer elements located several kilobases away from the promoter has been recognized for many years; however, a precise molecular understanding of any of these multiprotein interactions is still lacking (see Carter et al. 2002). In prokaryotes, although some gene regulators, such as DeoR (Dandanell et al. 1987) and NtrC (Reitzer and Magasanik 1986) are able to work over large distances, most regulatory protein binding sites lie within 300 bp of the promoter (Gralla and Collado-Vides 1996). The discovery that the well studied CI protein of bacteriophage λ interacts over DNA distances of up to 3.8 kb (Révet et al. 1999; Dodd et al. 2001) therefore provides a unique opportunity for the characterization of long-range gene regulation. The wealth of available biochemical data and the relative ease of testing theoretical predictions have also made the λ system ideal for the development of models of gene regulatory networks (Shea and Ackers 1985; Arkin et al. 1998; Aurell et al. 2002), and an important tool in the emerging field of gene circuit engineering (Hasty et al. 2002). Information about the O_L–O_R interaction should contribute to the refinement of these models and tools.

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The CI protein of λ has been the subject of intensive genetic, molecular biological, biochemical, physical and structural study (for reviews, see Johnson et al. 1981; Ptashne 1998; Hochschild 2002; see also Bell et al. 2000). CI binds to two operator regions, O_R and O_L, located 2.4 kb apart on the phage chromosome, with each region containing three individual CI operators spaced ~two DNA turns apart (Fig. 1A). Each operator is contacted by a CI dimer, with dimers binding cooperatively to adjacent pairs of operators and preferentially occupying $O_R1.O_R2$ and $O_L1.O_L2$. This represses the early lytic promoters P_R and P_L and activates the weak promoter for the *cI* gene, P_{RM} (Johnson et al. 1981; Ptashne 1998). At high concentrations, CI can also repress P_{RM} by occupying O_R3 (negative autoregulation; Maurer et al. 1980).

The CI protein consists of two domains joined by a protease sensitive linker of ~40 amino acids (Pabo et al. 1979). The N-terminal domain (NTD) is responsible for DNA binding and contains the amino acids that contact the σ subunit of RNA polymerase to activate P_{RM} (Nickels et al. 2002 and references therein). The C-terminal domain (CTD) is responsible for CI self-assembly and cooperative DNA binding (Bell et al. 2000). Full-length CI associates to tetramers and octamers in the absence of DNA, although such oligomers are rare in solution at physiological CI concentrations (Senear et al. 1993). Genetic studies have shown that cooperative DNA binding of CI dimers is mediated by a group of residues in the CTD (Bell et al. 2000 and references therein). It is likely

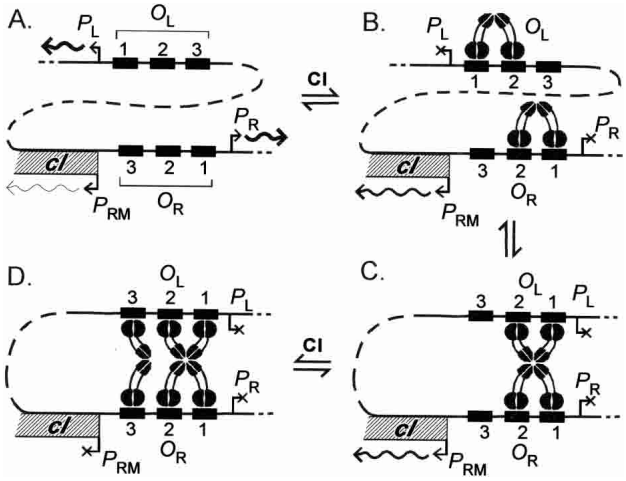

Figure 1. Model of CI regulation with long-range DNA looping. Cartoon depicting the major predicted CI:DNA complexes at O_L and O_R on the λ chromosome and their effects on transcription as CI concentration increases (*A* to *B*, *C* to *D*).

that the same CTD interface is used for tetramerization on DNA and in solution, because mutations that disrupt cooperativity on the DNA also disrupt tetramerization in solution (Burz and Ackers 1996), and the amino acids involved in cooperativity interact at the dimer–dimer interface in the crystal structure of the CTD tetramer (Bell et al. 2000).

The CI tetramerization reaction is capable of mediating the interaction of CI dimers bound to DNA sites spaced up to a few helical turns apart (Hochschild and Ptashne 1986) but becomes undetectable at distances beyond 20 turns (A. Hochschild and M. Ptashne, unpubl.). However, Révet et al. (1999) showed that CI dimers bound to *pairs* of adjacent operators could interact over much larger distances in vitro and in vivo. Repression of P_R by CI binding to O_R1 and O_R2 was enhanced fourfold by the presence of O_L1 and O_L2 (but not O_L1 alone) at a distance of 3.6 kb. Révet et al. (1999) thus proposed that the interaction involved the formation of a CI octamer (Fig. 1C). Although this has not been tested directly, octamerization of DNA-bound CI is consistent with the ability of CI to octamerize in solution (Senear et al. 1993) and with the crystal structure of the CTD octamer (Bell and Lewis 2001).

Whether the improvement in P_R repression caused by the O_L–O_R interaction is physiologically significant is not clear. However, repression of P_{RM} by CI in a λ lysogen is dependent on the presence of O_L. This negative autoregulation by CI limits the CI concentration in the lysogen and facilitates efficient induction of the prophage in response to UV light (Dodd et al. 2001). To explain this effect of O_L on P_{RM} repression, it was proposed that the CI-mediated O_L–O_R loop juxtaposes O_R3 and O_L3 so that a CI dimer bound at O_L3 (a higher-affinity binding site than O_R3) can assist a CI dimer to bind at O_R3 and repress P_{RM} (Fig. 1D; Dodd et al. 2001). Although this model is plausible and provides a rationale for retention of O_L3 in the evolution of λ, it has not been tested.

In this paper, we confirm a number of aspects of the proposed model for CI-mediated negative autoregulation (see Fig. 1D). Specifically, we show that (1) O_L3 is necessary for repression of P_{RM} at physiological CI concentrations, (2) the primary role of O_L3 is to foster CI binding at O_R3 and so provide CI negative autoregulation, (3) the interaction between O_R3 and O_L3 is mediated by the CI CTD, and (4) the long-range O_R3–O_L3 interaction only occurs in the presence of the other four operators. Finally, we incorporate the proposed CI interactions between O_R and O_L into a physicochemical model of CI regulation and find that a good approximation to our in vivo promoter reporter data is achieved by using some fairly simple assumptions about the energetics of the long-range interactions.

Results

Mutation of O_L3 *blocks CI repression of* P_{RM}

In the model shown in Figure 1D, CI binding to O_R3, and thus repression of P_{RM}, is enhanced by a cooperative interaction with CI bound at O_L3. To test this, we eliminated CI binding to O_L3 by altering four base pairs in the operator (creating the O_L3-4 mutation). The predicted effect of the changes on CI binding is a $\Delta\Delta G$ of +10.3 kcal/mole (Sarai and Takeda 1989).

The O_L3-4 mutation was introduced into the chromosomal P_{RM} reporter construct shown in Figure 2, in which O_L ($O_L1.O_L2.O_L3$) is located downstream of *lacZ* expressed under the control of $P_{RM}.O_R$ ($O_R3.O_R2.O_R1$). This P_{RM}.*lacZ* fusion construct contains the +62 to –123 region of P_{RM} with an O_L–O_R spacing of 3.8 kb. A range of CI concentrations was supplied to the reporters from a plasmid that directed the synthesis of CI under the control of an IPTG-inducible promoter. The CI levels relative to those in a wild-type λ lysogen (wild-type lysogenic units, WLU) were determined previously (Dodd et al. 2001).

When O_L3 and O_R3 were intact, P_{RM} was activated at low CI concentrations but became repressed at higher CI concentrations (Fig. 2A). As expected, repression was lost when O_R3 carried the *r1* mutation or when O_L was not present (Dodd et al. 2001). (As seen previously, maximal P_{RM} activation was ~35% greater in the absence of O_L than in its presence. It seems that octamer formation slightly inhibits the ability of the CI dimer bound at O_R2 to activate P_{RM}). CI repression of P_{RM} was also abolished by the O_L3-4 mutation. Thus, the O_L3 CI binding site, located 3.8 kb away from P_{RM}, was necessary for repression of P_{RM} by physiological levels of CI.

O_L3 *is not needed for CI repression of* P_R

These results support the idea of a specific interaction between O_L3 and O_R3 for CI repression of P_{RM}. However, an alternative possibility is that the CI dimer at

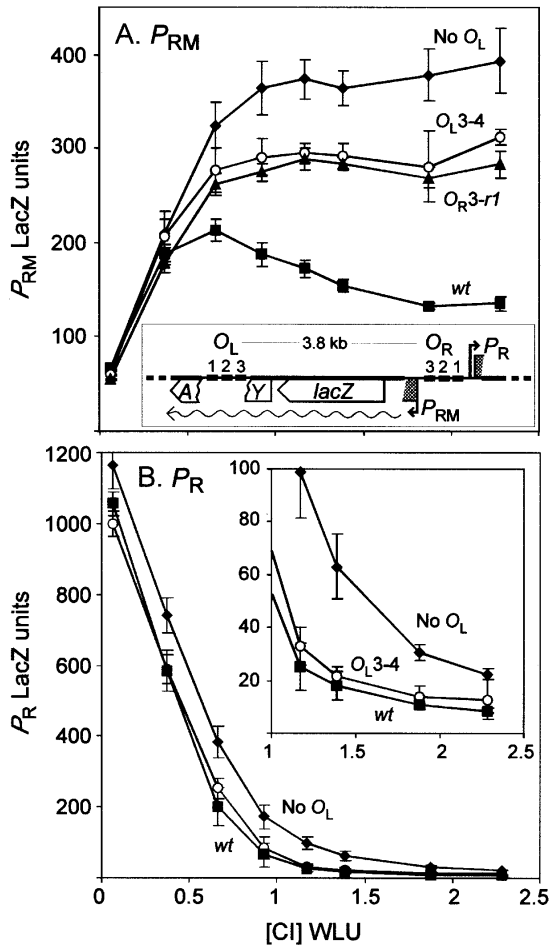

Figure 2. O_L3 is needed for efficient CI repression of P_{RM} but not P_R. Activities of P_{RM}::*lacZ* (*A*) and P_R::*lacZ* (*B*) operon fusions over a range of CI concentrations (in wild-type lysogenic units, WLU) supplied using plasmids pZC320*cI* and pUHA1 (providing *lac* repressor) with IPTG (0 to 200 μM). The *wt* P_{RM} reporter fusion is depicted (*A*); in the P_R reporters the orientation of the O_R fragment is reversed. Each reporter carried three operators at O_R; these were wt except for the O_R3-*r1* P_{RM}::*lacZ* fusion (O_R3-*r1*). Downstream of *lacZ* there was either no λ sequences (no O_L) or a 70-bp wt $O_L1.O_L2.O_L3$ fragment (*wt*) or one carrying the O_L3-4 mutation (O_L3-4). *Inset* in (*B*) expands the rightmost portion of the P_R graph. Error bars indicate 95% confidence limits ($n > 5$).

O_L3 plays a more indirect role in facilitating P_{RM} repression, for example by interacting with the CI dimer bound at O_R1 and thereby enabling the CI dimer bound at O_R2 to cooperate with a dimer bound at O_R3. We investigated the possibility that the CI dimer bound at O_L3 interacts with CI bound at O_R1 or O_R2 by testing whether the O_L3-4 mutation interfered with CI repression of P_R.

The O_R fragment in the reporter construct of Figure 2A was reversed so that *lacZ* was expressed from P_R (fragment is +42 to −143 of P_R). Assays in the presence of a range of CI concentrations showed that CI repression of P_R was most efficient when wild-type O_L was present (Fig. 2B). Removal of O_L weakened repression by up to fourfold, depending on the CI concentration, consistent with the finding of Révet et al. (1999). However, the O_L3-4 mutation weakened repression of P_R only slightly, indicating that the O_L–O_R loop was not disrupted.

These results show that, despite CI binding at O_L3 being critical for repression of P_{RM}, it does not contribute strongly to the O_L–O_R interaction, supporting the idea of a specific interaction between O_L3 and O_R3.

Whole phage studies—the role of O_L3 *in CI autoregulation and prophage induction*

The function of O_L3 in λ development has been a puzzle because the binding of either CI or Cro to this operator should not affect P_L activity (Johnson et al. 1981). Our results showing a need for O_L3 in CI repression of P_{RM} now suggest a role for O_L3, because repression of P_{RM} is necessary for efficient switching into lytic development in response to UV (i.e., prophage induction; Dodd et al. 2001). To test whether or not O_L3 actually facilitates prophage induction, the O_L3-4 mutation was introduced into wild-type λ to give λO_L3-4. The mutation had no detectable effect on plaque morphology or phage production kinetics after infection (data not shown). We measured CI levels in NK7049 monolysogens of λ*r1* (carries O_R3-*r1* mutation) and λO_L3-4 using our gel mobility shift assay for CI DNA binding activity (Dodd et al. 2001). Combining these and previous gel shift assay results gave estimated CI levels of 2.8 WLU (95% confidence limits: 2.51–2.99, $n = 5$) and 3.0 WLU (95% confidence limits: 2.94–3.14, $n = 4$) for the λ*r1* and λO_L3-4 lysogens, respectively. Thus, the O_L3-4 and O_R3-*r1* mutations each caused a similar increase in the lysogenic CI concentration, confirming that O_L3 is needed for CI repression of P_{RM} in the native λ context.

We then compared the UV-inducibility of the wild type and mutant prophages by measuring the fraction of lysogens induced versus dose of UV (Dodd et al. 2001). The λO_L3-4 lysogen displayed a defect in prophage induction very similar to that of the λ*r1* lysogen, with only 25%–33% as many lysogens induced compared to the wild-type control at three different UV doses (data not shown). This defect is presumably due to the high level of CI in the lysogens. It was possible that the O_L3-4 mutation was somehow acting through an effect on P_L or its regulation, but we found using P_L.*lacZ* reporters that the basal activity and CI repressibility of P_L were not substantially affected by the O_L3-4 mutation (data not shown).

Thus, our results provide an explanation for the retention of the third operator at O_L, indicating that the primary role of O_L3 is to provide for CI negative autoregulation in order to limit the CI concentration in the lysogenic state and allow efficient switching to lytic development.

Localizing CI CTDs at O_L3 *is sufficient to facilitate repression of* P_{RM}

According to our model, the role of O_L3 in P_{RM} repression is to localize the CTDs of a dimer for contact with

the CTDs of a CI dimer bound at O_R3 (Fig. 1D). The P22-λ hybrid repressor of Whipple et al. (1994) carries the DNA-binding NTD of P22 repressor (residues 1–94) fused within the linker region to the CTD of wild-type (wt) λ repressor (residues 112–236). This hybrid protein is functional for binding to P22 operators and for cooperative interactions with dimers of the same type and with dimers of λ repressor (Whipple et al. 1994). Accordingly, we predicted that this hybrid repressor would be able to substitute functionally for wt λ CI if bound to the DNA at the position of O_L3.

To test this prediction, we replaced O_L3 on the chromosomal P_{RM} reporter constructs with the P22 O_R1 operator (Poteete et al. 1980). λ operators are 17-bp-long, and the center-to-center spacing of O_L2 and O_L3 is 20 bp; in the replacement, the spacing between O_L2 and the 18-bp P22 operator was 20.5 bp. The P22-λ hybrid repressor was supplied to these reporters from a plasmid that directs the synthesis of an amount corresponding to roughly 10 lysogen's worth of λ repressor. The lysogenic level of wt λ repressor was supplied in all cases from a λ prophage (λ*att80*) integrated at the φ80 attachment site. In addition, because the reporter phages carry the immunity region of phage 21 (*imm21*), the phage 21 repressor was also present. The phages P22 and 21 are homo-immune; the P22 repressor can bind to phage 21 operators and 21 repressor can bind to P22 operators (Ballivet et al. 1977, 1978), and the O_R1 operators of these phages are identical (Poteete et al. 1980).

Repression of P_{RM} was measured as P_{RM}–*r1* LacZ activity (= unrepressed) divided by the activity of wt P_{RM}. In the absence of the hybrid repressor and with wt O_L3, P_{RM} was repressed 2.5-fold (Fig. 3, line 1). As expected, replacement of λO_L3 with the P22 operator prevented this repression of P_{RM} by λ repressor (Fig. 3, line 4). (Note that in the absence of repression a P_{RM} 'repression' value of 1.1 is obtained because the *r1* mutation improves P_{RM} activity by about 10%; Dodd et al. 2001). Because we expect the resident reporter-derived 21 repressor to occupy the O_L3 position in this construct, this result indicates that repression of P_{RM} is not enhanced by the binding of just any protein at the O_L3 position. The presence of the hybrid repressor restored P_{RM} repression in the case of the reporter construct with the P22 operator at the position of O_L3 (2.3-fold repression; Fig. 3, line 5), consistent with the idea that a hybrid repressor dimer is able to bind to the P22 operator using its P22 NTDs and simultaneously contact λ CI dimer bound at O_R3 using its λ CTDs (diagram of Fig. 3). Repression was almost as strong as in the native λ situation (Fig. 3, line 1), indicating that the lysogenic concentration of the 21 repressor does not compete significantly with the ~10 lysogen's worth of hybrid repressor for binding to the P22 operator at O_L3.

This experiment shows that repression of P_{RM} does not require a particular DNA sequence at O_L3 or a particular DNA binding domain bound at O_L3. Instead, effective repression can be achieved simply by tethering a pair of λ CI CTDs at O_L3 for interaction with the CTDs of a CI dimer bound at O_R3.

	Operator at O_L3 position	Repressors present: λ	φ21	P22-λ hybrid	P_{RM} repression	
1.	λ	+	+	–	2.5	(771/310)
2.	λ	+	+	wt	2.2	(834/379)
3.	λ	+	+	D197G	2.5	(789/315)
4.	P22	+	+	-	1.1	(857/798)
5.	P22	+	+	wt	2.3	(874/388)
6.	P22	+	+	D197G	1.3	(915/716)

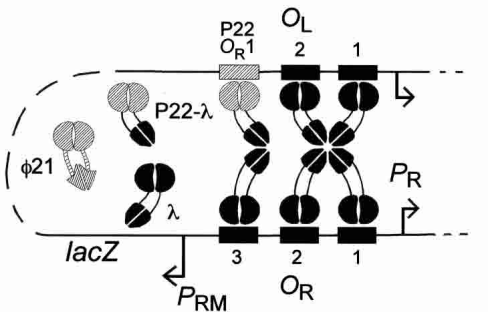

Figure 3. CI CTDs at O_L3 assist in repression of P_{RM}. The reporter constructs were as in Fig. 2, except that in some cases the λ O_L3 operator was substituted by a P22 O_R1 operator (gray box). The cartoon shows the interactions expected for line 5. λ repressor was supplied from the λ*att80* prophage, and the φ21 repressor was from the λ*imm21* reporter prophage itself. The P22-λ hybrid repressor (about 10 WLU) was supplied (or not) from plasmid pFW7-280Δ (or parent plasmid pLR1ΔcI) with 5μM IPTG. The P22-λ hybrid repressor bearing the D197G substitution was supplied from pFW7-280Δ D197G, a derivative of pFW7-280Δ. Plasmid pAD325 was the source of the *lac* repressor. P_{RM} repression values are calculated by dividing the P_{RM} *lacZ* units for reporters carrying the *r1* mutation in O_R3 (unrepressible) by the units for reporters where O_R3 is wt. Shown are the average results of duplicate assays performed in a single representative experiment. The cells containing either pFW7-280Δ (encoding the hybrid repressor) or pLR1ΔcI (encoding no repressor) were assayed on four separate occasions with similar results, and the cells containing pFW7-280Δ D197G were assayed on two separate occasions with similar results.

Figure 2 showed that repression of P_{RM} was lost when either O_R3 or O_L3 was mutated to prevent CI binding. The data in Figure 3 show that a similar loss of repression occurs when both O_R3 and O_L3 are mutated, indicating that the two mutations are not acting independently, but rather to disrupt the same repressive mechanism. The P_{RM} activity of the O_L3-P22 O_R3-*r1* double mutant (857 units, Fig. 3, line 4) was similar to the activities seen with either single mutant (Fig. 3, line 1, $O_L3^+O_R3$-*r1* = 771 units; line 4, O_L3-P22 O_R3^+ = 798 units).

We wished to test the inference that interaction between CI dimers at bound at O_R3 and O_L3 involves the same region of the CI CTD that mediates cooperative binding of pairs of dimers to operators separated by two or a few helical turns. To do this, we introduced the amino acid substitution D197G into the λ CI CTD on

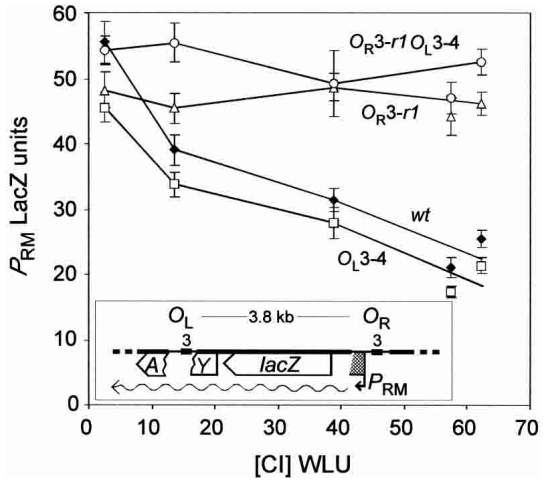

Figure 4. O_R3 and O_L3 do not interact in the absence of the other operators. Response to CI of P_{RM}::*lacZ* operon fusions bearing single O_L3 and O_R3 operators at O_L and O_R. The structure of the reporter fusions is shown in the *inset*. The O_R3-*r1* and O_L3-4 mutations were used to block CI binding to either or both operators. CI was supplied using plasmids pZE15*cI* and pUHA1 (0–500 μM IPTG). Error bars indicate 95% confidence limits (n = 8).

the P22-λ hybrid repressor. This substitution abolishes cooperativity between CI dimers but does not affect DNA binding or repressor dimerization (Whipple et al. 1994, 1998). In addition, residue D197 lies on the interface between repressor dimers in the crystal structure of the CI C-terminal domain tetramer (Bell et al. 2000). The P22-λ D197G repressor, unlike the wild-type hybrid, was unable to assist repression of P_{RM} (Fig. 3, line 6), supporting the idea that the long-range interaction between O_L3 and O_R3 is mediated by the same interface used for short-range dimer–dimer interactions.

The O_L3–O_R3 interaction alone is insufficient for CI autoregulation

We hypothesized that the CI-mediated interaction between O_L3 and O_R3 occurs efficiently only because the formation of a CI octamer linking $O_R1.O_R2$ and $O_L1.O_L2$ juxtaposes O_L3 and O_R3 suitably for the binding of a CI tetramer. We tested this hypothesis by examining whether O_L3 alone was able to assist CI binding to O_R3 alone to repress P_{RM}. The P_{RM} reporter construct shown in Figure 4 contains O_L3 and O_R3 spaced at 3.8 kb (precisely the same distance as in the Fig. 2 constructs) but does not carry O_L1, O_L2, O_R1, or O_R2. There was no activation of P_{RM} in these reporters because O_R2 is absent. We found that O_R3^+ versions of this reporter were only slightly repressed at 4 CI WLU (using pZC320*cI*, data not shown), so we introduced a higher range of CI concentrations (up to 60 WLU) by using a high-copy CI expression plasmid, pZE15*cI* (Dodd et al. 2001). We found that repression of P_{RM} did occur at these high CI levels as long as O_R3 was intact (Fig. 4). Because O_L3 is a considerably stronger binding site for CI than O_R3 (see Table 1) and because it is clear that O_R3 is occupied at these concentrations, we are confident that O_L3 was also occupied. However, there was no enhancement of P_{RM} repression by the presence of an intact O_L3 at 3.8 kb (Fig. 4). Thus, cooperativity at this distance between CI bound at O_L3 and O_R3 requires the presence of the other λ operators.

Physicochemical modeling of the CI-O_L-O_R system

Statistical thermodynamic analyses of CI DNase I footprint titrations have allowed resolution of the free energies for interaction of CI with each of the six operators and also for the cooperative interactions between CI bound within the same set of operators (Table 1). However, previous analyses have not taken into account long-range interactions between O_L and O_R. In order to validate our model of CI regulation and also possibly to derive estimates of the free energies of the long-range interactions, we modified the statistical thermodynamic approach of Shea and Ackers (1985) to incorporate the

Table 1. *Parameters used in physicochemical modeling*

Operator[a]	ΔG (kcal/mole)	Operator	ΔG (kcal/mole)
O_R1	−13.2	O_L1	−13.8
O_R2	−10.7	O_L2	−12.1
O_R3	−10.2	O_L3	−12.4
O_R1-O_R2 coop	−3.0	O_L1-O_L2 coop	−2.5
O_R2-O_R3 coop	−3.0	O_L2-O_L3 coop	−2.5
O_R1-O_R3 coop	0	O_L1-O_L3 coop	0
O_R1-O_R2-O_R3 coop	−3.0	O_L1-O_L2-O_L3 coop	−2.5
O_R3 (*c12*)[b]	−11.0	O_L3-4[c]	−6.2
O_R3 (*r1*)[b]	−7.3		

Parameter	Value	Promoter	LacZ units[h]
K_{dim}[d] (M^{-1})	6.7×10^7 (ΔG_{dim} = −11.1)	P_R basal	1056
K_{NS}[e] (M^{-1})	2.5×10^4 (ΔG_{NS} = −6.2)	P_R repressed	2
[NS][f]	6.76×10^{-3} M	P_{RM} basal	45
$[CI]_{lysogenic}$[g]	3.7×10^{-7} M	P_{RM} activated	360 (265)
		P_{RM} repressed	0.5

[a] O_R values from Koblan and Ackers (1992); O_L values from Senear et al. (1986).
[b] Calculated from ΔΔG values of Sarai and Takeda (1989); see Dodd et al. (2001) for sequences.
[c] Set as equal to nonspecific binding.
[d] CI dimerization (Koblan and Ackers 1991).
[e] Affinity for nonspecific DNA; see text for details. K_{NS} is a tool to align in vitro and in vivo repressor concentration scales, and we ascribe no particular significance to its value.
[f] Concentration of nonspecific sites from *E. coli* genome size (4.6×10^6 bp/cell) and 1 molecule/cell = 1.47 nM (Donachie and Robinson 1987).
[g] Calculated from the data of Reichardt and Kaiser (1971) and 1 molecule/cell = 1.47 nM.
[h] Wild-type values; value in parentheses for looped configuration. P_R values for mutants (basal/repressed): O_L3-4 998/2, no O_L 1164/2. P_{RM} values for mutants (basal/activated/repressed): *c12* 37/240(180)/0.5, *r1* 47/380(280)/0.5.

O_L–O_R interaction. We then tested whether this new statistical thermodynamic model, with suitable values for the long-range interaction parameters, could simulate our P_{RM} and P_R reporter data.

The ΔG for each configuration of the 64 possible species for CI occupancy of O_R and O_L (six operators, each unoccupied or occupied by CI) can be calculated from the free energies listed in Table 1. We modeled the O_L–O_R interaction by also considering looped-unlooped equilibria, introducing nine looped configurations and two new cooperative free energy terms, ΔG_{oct} and ΔG_{tet}. DNA looping was permitted for any species in which CI was bound to an adjacent pair of operators at O_L *and* to an adjacent pair of operators at O_R (four different four-dimer species, four different five-dimer species, and the single six-dimer species), and the free energy for each such looped configuration was obtained by adding the ΔG_{oct} cooperativity term to the free energy calculated for the unlooped configuration. This cooperativity term reflects a net free energy change due to octamerization of CI bound across O_R–O_L, together with the cost of formation of a DNA loop (Fig. 1B,C). The ΔG_{tet} cooperativity term was added only in the case where all six operators were occupied (applied in addition to ΔG_{oct}). This cooperativity term reflects an overall favorable free energy change due to tetramerization between CI bound to the remaining operator at O_L and to the remaining operator at O_R when a CI octamer is already present. Based on the experiment of Figure 4, which showed that a single operator at O_L and a single operator at O_R do not cooperate, ΔG_{tet} was not applied to species in which octamer formation could not occur.

To generate curves that would simulate our LacZ reporter data (LacZ activity as a function of CI concentration), we assigned LacZ activities for P_R and P_{RM} for each operator configuration, based on the LacZ activities measured with our reporter constructs (derepressed or fully repressed for P_R; basal, activated, or repressed for P_{RM}; Fig. 2; Dodd et al. 2001). The LacZ values used are listed in Table 1; further details are given in Materials and Methods. CI concentrations were converted to lysogenic units by using a lysogenic CI concentration of 370 nM (Table 1). Initially, we found that the simulations gave CI activities that were ~10-fold more effective than expected from the reporter data. Binding of CI to nonspecific DNA in vivo should lower the concentration of CI available to interact with specific sites, and we were able to align theoretical repression curves with in vivo repression data by considering this nonspecific binding (Materials and Methods). The parameter K_{NS} (the affinity of CI for a single nonspecific site) was adjusted to give a reasonable fit to reporter data for P_{RM} and for P_R in the absence of CI binding to O_L.

Reasonable fits to all *lacZ* reporter data for wild-type and mutant P_{RM} and P_R promoters in the presence of O_L were obtained with free energy values of –0.5 kcal/mole for ΔG_{oct} and –3 kcal/mole for ΔG_{tet}. Figure 5A shows the simulations for P_{RM} when O_R is wild-type or carries mutations in O_R3 that abolish (*r1*) or improve (*c12*) CI binding. Figure 5B shows the simulation of the P_R data from Figure 2B. There is a reasonable fit between data and theory at higher CI concentrations, reproducing the weakening of P_R repression by the loss of O_L and also the very slight effect of the O_L3-4 mutation. However, for reasons we do not understand, the theoretical curves predict stronger repression of P_R than we observed at CI concentrations below 1 WLU.

The effects of alterations in ΔG_{oct} and ΔG_{tet} on the fit of the simulations with the data are shown for wild-type P_{RM} in Figure 5C,D. With ΔG_{tet} held at –3 kcal/mole, lowering ΔG_{oct} below –0.5 kcal/mole produced a more sensitive CI activation of P_{RM}, whereas increasing ΔG_{oct} values above –1 kcal/mole weakened CI repression of P_{RM} (Fig. 5C). Setting ΔG_{oct} to +5 kcal/mole, effectively eliminating DNA looping, showed the lack of P_{RM} repression that would be expected for a CI mutant specifically defective in octamerization. Lowering ΔG_{tet} while holding ΔG_{oct} at –0.5 kcal/mole also increased CI repression of P_{RM} (Fig. 5D).

Figure 5E shows the population distribution of the 64 different operator occupation species (looped and unlooped configurations are combined) as a function of total repressor concentration, calculated using ΔG_{oct} = –0.5 and ΔG_{tet} = –3 kcal/mole. With these cooperativities, the four-dimer and five-dimer species that are able to form loops are in the looped configuration 69% of the time, whereas the fully liganded R123L123 species is 99.7% looped. Although the model allows the ΔG_{oct} cooperativity term for all potential octameric species, those species able to form an R12L12 octamer account for at least 90% of the possible octamer-containing species at any CI concentration.

Strikingly, despite the large number of possibilities, only five CI-bound species achieve more than a 10% share of the total fraction at any CI concentration (Fig. 5E). At the lysogenic CI concentration, three species make up 90% of the total: the R12L12 (~40%), the R12L123 complex (~20%), and the fully occupied R123L123 species (~30%); with ~72% of the total being the looped forms of these species. Remarkably, the lysogenic concentration occurs at a region of the graph at which the R12L12 and R123L123 curves have steep and opposite slopes, such that small fluctuations in CI concentration would produce large changes in the ratio of activated to repressed P_{RM}. Thus the lysogenic state seems to be poised for maximal responsiveness in CI negative autoregulation. Figure 5F gives the distribution of species calculated for the case where looping between O_R and O_L is effectively absent (ΔG_{oct} = +5). The major effect is the change in the fractions of the three lysogenic species, with the R123L123 species populated only at repressor levels well above the lysogenic concentrations, and only the two P_{RM}-activated species prevalent at physiological CI concentrations.

Discussion

The O_L–O_R *model*

Our results support the model depicted in Figure 1 describing the CI interactions that regulate transcription from the O_L–O_R region.

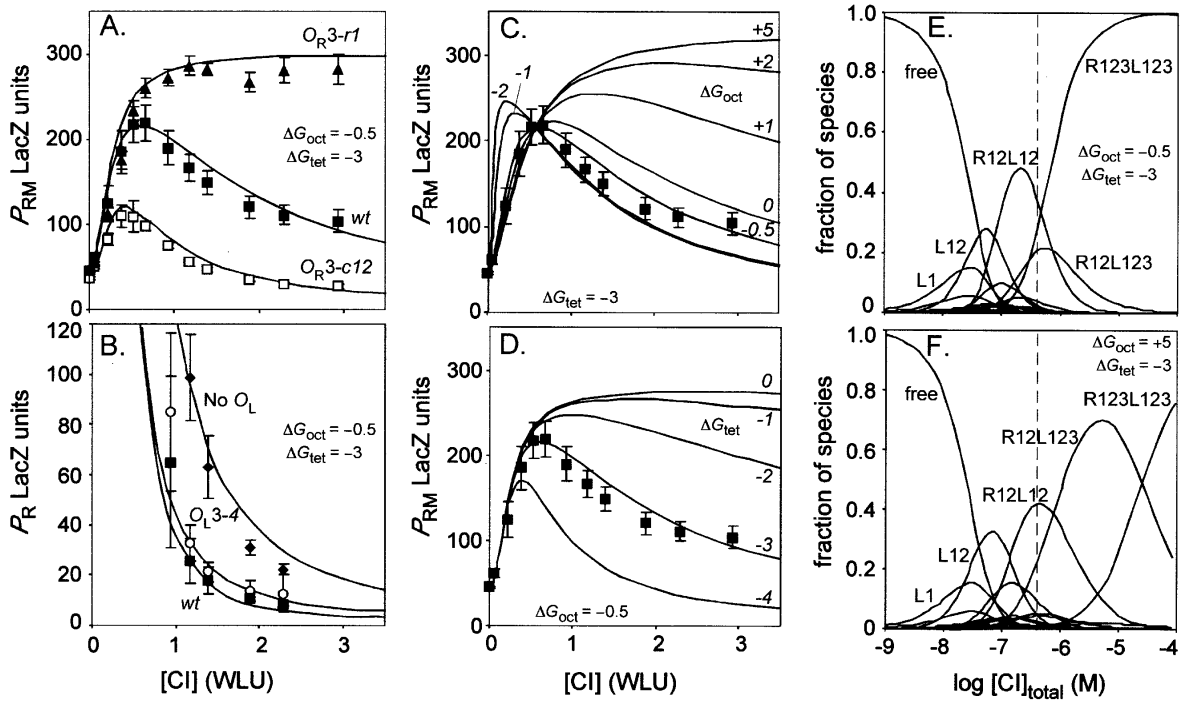

Figure 5. Physicochemical modeling of CI regulation. (*A–D*) Simulation of P_{RM} and P_R *lacZ* reporter data using a physicochemical model incorporating the long-range CI interactions (see text). Simulations (solid lines) using ΔG_{oct} = –0.5 kcal/mole and ΔG_{tet} = –3.0 kcal/mole for (*A*) *wt* P_{RM} or P_{RM} carrying the *r1* or the *c12* mutation in O_R3 (O_L present; data from Dodd et al. 2001); (*B*) the P_R reporter data of Fig. 2B. (*C,D*) The effect of varying ΔG_{oct} from –2 to +5 kcal/mole with ΔG_{tet} fixed at –3.0 kcal/mole (*C*) or varying ΔG_{tet} from –4 to 0 kcal/mole with ΔG_{oct} fixed at –0.5 kcal/mole (*D*) on the simulation of the *wt* P_{RM} data. (*E,F*) The fraction of each of the 64 possible CI-operator species (looped and unlooped fractions of each species combined) predicted by the model over a range of CI concentrations, in the presence (*E*; ΔG_{oct} = –0.5, ΔG_{tet} = –3.0 kcal/mol) or effective absence (*F*; ΔG_{oct} = +5, ΔG_{tet} = –3.0 kcal/mol) of the long-range interactions. Minor species are not labeled; the dashed line shows the lysogenic CI concentration.

First, O_L3 was shown to be necessary for CI repression of P_{RM} at physiological CI concentrations. Blocking binding of λ CI to O_L3, either by the O_L3-4 mutation (Fig. 2A) or by substitution with a P22 operator (Fig. 3), eliminated repression of P_{RM} by lysogenic CI concentrations in reporter constructs in which O_L was located 3.8 kb away from O_R. The requirement for O_L3 in CI negative autoregulation and efficient prophage induction was confirmed in the native phage context where O_L3 and O_R3 are separated by 2.4 kb. Because O_R3 is necessary for repression of P_{RM} at physiological CI concentrations (Fig. 2A) and is sufficient for repression at high CI concentrations (Fig. 4) and because CI binding at O_L3 is unable to repress P_{RM} directly (Fig. 4), we conclude that O_L3 works by fostering CI binding at O_R3.

Second, the proposal that O_L3 facilitates CI binding at O_R3 through a direct interaction between the dimers bound at O_L3 and O_R3 was supported by the finding that O_L3 did not contibute significantly to the enhancement of CI repression of P_R by O_L, indicating that CI at O_L3 does not interact significantly with CI bound to O_R1 or O_R2 (Fig. 2B). The conclusion that CI bound at O_L3 interacts primarily with CI bound at O_R3 was strengthened by the physicochemical modeling, which indicated (assuming that ΔG_{oct} is the same for all octamers) that L12R12 is the favored octameric species at lower CI concentrations, with the O_L3 and O_R3 sites becoming filled at higher CI concentrations.

Third, the idea that the sole role of the O_L3-bound CI dimer in facilitating P_{RM} repression is to make a favorable protein–protein contact with the dimer bound at O_R3 was supported by the demonstration that the CI dimer bound at O_L3 could be functionally replaced by a hybrid repressor bearing the λ CI CTD but a heterologous DNA-binding domain. Specifically, O_L3 was replaced with a P22 operator, and the hybrid repressor bore the P22 repressor DNA-binding domain (Fig. 3). Moreover, the protein–protein contact of the λ CI CTDs was shown to be similar to that providing short-range cooperativity between CI dimers, because P_{RM} repression was lost when the λ CI CTD of the hybrid repressor carried an amino acid substitution that prevents short-range cooperativity.

Fourth, O_L3 and O_R3 did not cooperate in the absence of the other operators, even at CI concentrations where O_R3 and, presumably, O_L3 were occupied (Fig. 4). Thus, the energetic benefit of the O_R3-O_L3 interaction must not outweigh the energetic cost of DNA looping. This supports the idea that the $O_R1.O_R2$–$O_L1.O_L2$ interaction, which mediates repression of P_R and P_L and activation of P_{RM} at low CI concentrations, occurs first and 'pays' the cost of the DNA looping. Thus, the looped

structure that is formed by the interaction of CI dimers bound at $O_R1.O_R2$ and $O_L1.O_L2$ presumably juxtaposes O_L3 and O_R3 so that CI dimers bound at those sites can interact with little additional DNA conformational change.

Fifth, the regulatory model (Fig. 1) was supported by a thermodynamic analysis that incorporated the long-range interactions and was able to simulate P_{RM} and P_R reporter data reasonably well. We obtained an estimate of ΔG_{oct} = –0.5 kcal/mole for CI octamerization with a 3.8-kb DNA loop and an estimate of ΔG_{tet} = –3.0 kcal/mole for the O_R3–O_L3 CI tetramerization reaction, a value similar to short-range cooperativities between CI dimers (Koblan and Ackers 1992). Although these cooperativities are small, they are able to significantly improve CI repression of the P_R lytic promoter and give repression of P_{RM} at the lysogenic CI concentration. These long-range cooperativities appear to position the lysogenic state ideally for CI negative autoregulation, such that small changes in CI concentration should produce relatively large compensatory changes in P_{RM} activity.

It is important to note that the values for the long-range cooperativity terms (ΔG_{oct} and ΔG_{tet}) used in our physicochemical model cannot provide structural information. Formally, ΔG_{oct} represents a stabilization of the four-dimer CI complex and ΔG_{tet} represents an additional stabilization of the six-dimer CI complex. Although plausible, it is not certain that CI is able to form an octamer while bound simultaneously to O_L and O_R. In theory, the four-dimer complex could be a pair of '*trans*' tetramers, for example, a CI tetramer linking O_L1 and O_R1 with another tetramer linking O_L2 and O_R2. Further structural and mutational studies will be necessary to resolve these uncertainties, and also uncertainties about the path that the DNA takes through the CI complex.

The role of DNA in long-range interactions

It is clear that the DNA plays a critical role in the interaction between CI dimers at O_L3 and O_R3, because the bringing together of these two DNA sites by the CI octamerization reaction is a prerequisite for the cooperative interaction to occur. Such assisted cooperativity is presumably a general feature of large nucleoprotein complexes.

What role does the DNA play in the initial long-range interaction of CI tetramers bound at $O_L1.O_L2$ and at $O_R1.O_R2$? The ΔG_{oct} cooperativity term represents the net free energy change when the four-dimer complex goes from the unlooped to the looped configuration. ΔG_{oct} is equal to $-RT \ln K_c$, where K_c is the equilibrium constant for the intramolecular cyclization reaction (Shore et al. 1981), and can be seen as the sum of two free energy changes, ΔG_P and ΔG_D. ΔG_P is a favorable change due largely to protein–protein association and is equal to $-RT \ln K_a$, where K_a is the equilibrium constant for the bimolecular association reaction for protein complexes on separate DNA molecules (Shore et al. 1981). In our case, this is the octamerization reaction for CI tetramers bound to O_L and O_R when the two sites are unlinked. ΔG_D is an unfavorable change due to bringing the two DNA sites together. For a DNA loop of this size, ΔG_D is expected primarily to reflect the entropic cost of the loss of freedom for the DNA due to the protein-induced restraint (Rippe 2001).

Neither ΔG_P nor ΔG_D are known for the long-range CI octamerization reaction. However, provisional estimates of their values indicate that the reaction should be unfavorable, suggesting that there are extra factors favoring the reaction. A provisional estimate for ΔG_P (–9.1 kcal/mol) can be obtained from the free energy of association of two CI tetramers in the absence of DNA, as determined by sedimentation equilibrium experiments (Senear et al. 1993). Octamerization of CI bound to short single-operator DNA fragments is only slightly less favorable (Rusinova et al. 1997). Unfortunately, data for CI association in the presence of double operator fragments are not available. Provisional estimates of ΔG_D can be obtained from modeling of in vitro DNA cyclization. Because $\Delta G_D = \Delta G_{oct} - \Delta G_P$, $\Delta G_{oct} = -RT \ln K_c$ and $\Delta G_P = -RT \ln K_a$ (see above), then $\Delta G_D = -RT \ln(K_c/K_a)$. The ratio K_c/K_a is the parameter j (Shore et al. 1981), which is the local concentration of one site on the DNA relative to another site on the DNA. Theoretical models have been developed to permit calculation of j over a large range of DNA separations, and these values fit reasonably well with observed effects of DNA length on the rate of cyclization of linear, naked DNA in vitro (Rippe 2001). We used the equation of Rippe to calculate j for a 3.8-kb separation as 1.1×10^{-8} M (Eq. 3: Kuhn length l = 100 nm, L_m = 0.34 nm per bp, d = 0; Rippe 2001), giving an estimate of ΔG_D = 11.3 kcal/mole. Note that, by this calculation, the DNA tether makes the concentration of O_L relative to O_R ~sevenfold higher than the concentration of an unlinked O_L site in the cell (~1.5×10^{-9} M; Table 1). Combining these provisional estimates for ΔG_P and ΔG_D gives a ΔG_{oct} value of +2.2 kcal/mole, considerably higher than our ΔG_{oct} value of –0.5 kcal/mole and a value that would make DNA looping and repression of P_{RM} inefficient (see Fig. 5C). How then can the observed efficiency of the O_L–O_R interaction in vivo be explained?

There are two reasons why ΔG_P might be more favorable than our provisional estimate of –9.1 kcal/mole. First, the in vitro conditions used for measuring CI octamerization may not adequately reflect in vivo conditions; CI may octamerize more readily in solution in vivo. (Note that, if CI octamers were to form too readily, then the $O_L1.O_L2$ and $O_R1.O_R2$ operators could each become occupied by octamers rather than tetramers, which would prevent DNA looping and cause a loss of regulation of P_{RM}.) Second, CI octamerization may be more favorable when CI dimers are bound to O_L and O_R DNA than when they are unbound. It is not unreasonable that CI DNA binding could favorably affect the octamerization reaction, though no such effect is seen with single operators (Rusinova et al. 1997).

The observed efficiency of the long-range CI looping reaction could be explained without any change to ΔG_P

if the long-range DNA interaction in vivo is some 80-fold more efficient than for naked, linear DNA in vitro. A number of in vivo factors seem capable of giving this degree of improvement (Rippe 2001). Long-range interactions may be aided by compaction of the DNA and by changes to DNA flexibility caused by the binding of nucleoid or other proteins. These parameters are represented in the equation for *j*, but reliable in vivo values are not known (Rippe 2001). Specific DNA bends, either intrinsic or due to bound proteins, if properly located between the interacting sites, can also improve DNA interactions. However, we do not expect this factor to be important in our case because the effect of specific bends is likely to be weak at large distances (Rippe 2001), and we also know that the O_L–O_R interaction occurs efficiently with two completely different intervening sequences (on the phage chromosome and on the *lacZ* reporter). A further in vivo factor thought to affect ΔG_D is DNA supercoiling. Supercoiling is expected to assist interactions between DNA sites because it should cause the DNA to wind into compact plectonemic structures in which separate DNA segments are more frequently in contact with each other. Physical simulations suggest that DNA supercoiling can improve *j* by two orders of magnitude (Vologodskii et al. 1992). This activity of supercoiling is also supported by observations of long-range DNA-based protein–protein interactions in vitro, in both eukaryotic (Barton et al. 1997) and prokaryotic systems (Liu et al. 2001), and in vivo in bacteria (see Scheirer and Higgins 2001 and references therein).

Dröge and Müller-Hill (2001) suggested that the location of O_L and O_R on the same DNA molecule is not important in the CI octamerization reaction and that O_L acts solely as a 'scaffold' to foster the assembly of a CI tetramer that would not otherwise form in the cell. They speculated that eukaryotic enhancers may act largely as scaffolds for the assembly of unique and potent protein complexes. Although O_L is in part acting in this way, we believe that the 'tethering' of O_L to O_R by the intervening DNA is critical. As discussed above, the linkage of O_L to O_R by 3.8 kb of DNA may improve their interaction ~560-fold compared to the interaction of unlinked sites within the cell (the product of a sevenfold increase in effective concentration over unlinked sites and an 80-fold improvement if ΔG_D is improved by 2.7 kcal/mole in vivo, see above). The idea that the tethering of protein binding sites can significantly increase their relative concentration in vivo, even at such large DNA distances, helps to explain the action of enhancers in more complex cells. The λ CI–O_L–O_R interaction provides a well defined and tractable experimental system for examining basic questions about the requirements for such long-range interactions on DNA in vivo.

Materials and methods

Strains and media

NK7049 (Δ*lacIZYA*)χ74 *galOP308* Str^R Su^- from Bob Simons (Simons et al. 1987) was the host for all LacZ assays and phage work. DH5α and MC1061 were hosts for recombinant DNA work. Cells were grown at 37°C in LB (Miller 1972) with addition of ampicillin (100 μg/mL except 30 μg/mL for pZC320) and kanamycin (50 μg/mL for pUHA1). λ*att80* was λ*imm* λ*h* ϕ80*att* ϕ80 from Gary Gussin (University of Iowa, Iowa City).

Construction of lacZ reporter fusions

In all constructions, the sequence of all inserted, mutagenized, or PCR-amplified regions of DNA incorporated into constructs was confirmed. Further details of the cloning procedures are available on request.

The construction of the λ*imm21* phage *lacZ* reporter vectors λRS45ΔYA (deletion in *lacY* and *lacA*; no O_L) and λRS45ΔYAO_L (carrying P_L^-.O_L1.O_L2.O_L3; depicted in Fig. 2A) is described in Dodd et al. (2001). λRS45ΔYAO_L.O_L3-4, λRS45ΔYAO_L3, λRS45ΔYAO_L3-4, and λRS45ΔYAO_L.O_L3P22 were made using the same approach. λRS45ΔYAO_L.O_L3-4 is the same as λRS45ΔYAO_L except that the sequence of O_L3 bears four changes (underlined) to inactivate CI binding: TATCAC<u>TAG</u>A G<u>T</u>TGGTT. λRS45ΔYAO_L.O_L3P22 is the same as λRS45ΔYAO_L except that the sequence of O_L3 (17 bp) and one bp to its right were substituted by the 18-bp P22 O_R1 operator: ATTAAGTG TTCTTTAAT. In λRS45ΔYAO_L3 and λRS45ΔYAO_L3-4, O_L1 and O_L2 have been removed, leaving the sequence G<u>GAGAT AATTTATCACCGCAGATGGTTAT</u> (or its O_L3-4 derivative) between the *BsrG*I and *SgrA*I sites (O_L3 and the remainder of O_L2 are underlined).

Plasmid-based *lacZ* fusions were constructed in pTL61T (Linn and St. Pierre 1990), transferred to the phage *lacZ* reporter vectors for insertion into the *E. coli* chromosome, and single-copy confirmed, as described in Dodd et al. (2001).

Construction of the P_{RM}*wt*::*lacZ* and P_{RM}*r1*::*lacZ* operon fusions in pTL61T is described in Dodd et al. (2001); the *r1* version carries the O_R3-*r1* mutation that inactivates CI binding. Construction of P_{RM}Δ(12)3-*wt*::*lacZ* and P_{RM}Δ(12)3-*r1*::*lacZ* operon fusions, in which O_R1 and O_R2 have been removed, was the same except that the cloned PCR product contained sequence from the –37 to +62 region of P_{RM} (4 bp of O_R2 remain). The pR*wt*::*lacZ* operon fusion was made the same way, except that the restriction sites added to the end of the PCR fragment were swapped, so that the fragment was inserted in the reverse orientation.

Expression and quantitation of CI in vivo

The CI expression plasmids pZC320*cI* (mini-F origin, Ap^R, P_{lac}^+::*cI*) or pZE15*cI* (colE1 origin, Ap^R, P_{lac}^+::*cI*), in combination with pUHA1 (supplies *lac* repressor), were used to supply IPTG-controlled levels of wt CI to *lacZ* reporters; cellular CI levels from pZC320*cI* were previously quantitated using a gel shift assay, by calibration against CI binding activites of extracts from λ lysogens (Dodd et al. 2001). Similar quantitation of CI levels produced by pZE15*cI* (with pUHA1) gave values of 2.5, 13.5, 38.9, 62.5, and 57.5 WLU (means of two extracts) for IPTG concentrations of 0, 50, 100, 200, and 500 μM, respectively.

The expression plasmid pFW7-280Δ (Whipple et al. 1998) was used to supply IPTG-controlled levels of the P22-λ hybrid repressor, and plasmid pLR1ΔcI (Whipple et al. 1994), which does not encode a repressor, served as the control plasmid. These plasmids are derived from pBR322 and provide resistance to ampicillin. Plasmid pAD325 (Derman et al. 1993) is a derivative of plasmid pACYC184 that contains the $lacI^q$ gene and provides resistance to chloramphenicol. Cells containing plasmids pFW7-280Δ (or pLR1ΔcI) and pAD325 were grown in the presence of 100 μg/mL carbenicillin and 25 μg/mL chloramphenicol.

LacZ assays

LacZ assays for Figures 2 and 4 were carried out using 96-well microtiter plates as described in Dodd et al. (2001), with the modification that for assaying repression of P_R, we found that subculturing and a second overnight incubation with IPTG before preparation of log phase cultures for assay was needed to remove residual LacZ. The method used for the LacZ assays of Figure 3 is essentially that of Miller (1972) modified as described in Whipple et al. (1998).

λO_L3-4 construction and characterization

An O_L-containing PCR fragment containing λ sequences from 35219 to 35858 was inserted into pBluescriptKS+ (Stratagene) to give pBS-O_L^+. The O_L3-4 mutation (see above) was introduced into pBS-O_L^+ using the Quikchange method (Stratagene). The mutant *Hpa*I:35260-*Pvu*I:35790 fragment was used to replace the equivalent wt fragment in pAP831, and the mutation was recombined in vivo from pAP831-O_L3-4 onto λ*imm434* (Dodd et al. 2001), to give λO_L3-4.

Physicochemical modeling

The relative probability of each operator configuration, *s,* is given as

$$f_s = \frac{\exp(-\Delta G_s / RT)[\mathrm{CI}_2]^i}{\sum_s \exp(-\Delta G_s / RT)[\mathrm{CI}_2]^i}$$

where ΔG_s is the sum of the contributions of all the free energies (see Table 1) for a given configuration (*s*), [CI_2] is the concentration of free CI dimers, and *i* is the stoichiometry of CI dimers bound in each *s* configuration (Shea and Ackers 1985). It was assumed that monomers and dimers of CI are in equilibrium in solution and that only dimers can bind DNA, either specifically or nonspecifically. Results are plotted in terms of total monomer concentration, taking into account nonspecific binding, according to

$$[\mathrm{CI}]_{\text{total, monomer}} = 2[\mathrm{CI}_2] + \sqrt{\frac{[\mathrm{CI}_2]}{K_{\text{dim}}}} + 2(K_{\text{NS}}[\mathrm{CI}_2][\mathrm{NS}])$$

where K_{dim} is the dimerization constant for repressor, K_{NS} is the binding constant describing nonspecific binding of repressor to DNA, and [NS] is the molar concentration of nonspecific sites in an *E. coli* cell (Table 1). K_{NS} was obtained by simulation (see text).

For each configuration, we assigned basal P_R activity when O_R1 and O_R2 were unoccupied, repressed P_R activity if either O_R1 or O_R2 were occupied, basal P_{RM} activity when O_R2 and O_R3 were unoccupied, activated P_{RM} activity when O_R2 but not O_R3 were occupied, and repressed P_{RM} activity when O_R3 was occupied. Basal promoter values were taken from reporter activities in the absence of CI; for P_{RM} (*wt*, *r1*, and c12), values were from Dodd et al. (2001), and for P_R (*wt* , O_L3-4, and no O_L), values were from Figure 2B (no IPTG). The activated P_{RM} values for the unlooped species were the maximal activities seen for P_{RM} (*wt*, *r1*, and c12) in the absence of O_L (Dodd et al. 2001). The activated P_{RM} values for the looped species were set 26% lower, in line with the effect of O_L on the maximal activity of P_{RM}-*r1* (Dodd et al. 2001). The values used are given in Table 1.

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