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

Ian B. Dodd,1,2 Keith E. Shearwin,1 Alison J. Perkins,1 Tom Burr,2 Ann Hochschild,2 and J. Barry Egan1

1Discipline of Biochemistry, School of Molecular and Biomedical Science, University of Adelaide, South Australia 5005, Australia; 2Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

Effective repression of CI transcription from P\text{RM} by the bacteriophage λ CI repressor requires binding sites \{O\text{L}1\} located 2.4 kb from the promoter. A CI tetramer bound to O\text{L}1,O\text{L}2 interacts with a tetramer bound near P\text{RM} (O\text{N}1,O\text{N}2), looping the intervening DNA. We previously proposed that in this CI octamer:DNA complex, the distant O\text{L}3 operator and the weak O\text{N}3 operator overlapping P\text{RM} are juxtaposed so that a CI dimer at O\text{L}3 can cooperate with a CI dimer binding to O\text{N}3. Here we show that O\text{L}3 is necessary for effective repression of P\text{RM} and that the repressor at O\text{L}3 appears to interact specifically with the repressor at O\text{N}3. The O\text{L}3-CI-O\text{N}3 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.

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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 multienzyme interactions is still lacking (see Carter et al. 2002). In prokaryotes, although some gene regulators, such as DeoR (Dandanell et al. 1987) and Ntc (Reiter 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\text{L}–O\text{N} interaction should contribute to the refinement of these models and tools.

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\text{L} and O\text{N}, 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\text{L}1,O\text{N}2 and O\text{L}1,O\text{N}2. This represses the early lytic promoters P\text{R} and P\text{E} and activates the weak promoter for the CI gene, P\text{RM} [Johnson et al. 1981; Ptashne 1998]. At high concentrations, CI can also repress P\text{RM} by occupying O\text{N}3 [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\text{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
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 [Senea 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 \( O_{L1}–O_{R1} \) interaction is physiologically significant is not clear. However, repression of \( P_{RM} \) by CI in a \( \lambda \) 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 \( O_{L1}–O_{R1} \) 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 \( \lambda \), 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 \( O_{L3} \) and \( O_{R3} \) is mediated by the CI CTD, and [4] the long-range \( O_{R3}–O_{R3} \) 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 repression 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 \( \Delta \)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 \( P_{RM} \) [\( O_{R3}O_{R2}O_{R1} \)]. 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 \( \lambda \) 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 \( O_{L3} \) 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 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 \( \lambda \) chromosome and their effects on transcription as CI concentration increases (A to B, C to D).
$O_3^3$ plays a more indirect role in facilitating $P_{RM}$ repression, for example by interacting with the CI dimer bound at $O_9^3$ and thereby enabling the CI dimer bound at $O_9^2$ to cooperate with a dimer bound at $O_9^3$. We investigated the possibility that the CI dimer bound at $O_9^3$ interacts with CI bound at $O_9^1$ or $O_9^2$ by testing whether the $O_3^3$-4 mutation interfered with CI repression of $P_{R}$.

The $O_9$ 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 $O_9$ was present [Fig. 2B]. Removal of $O_9$ weakened repression by up to fourfold, depending on the CI concentration, consistent with the finding of Révet et al. [1999]. However, the $O_3^3$-4 mutation weakened repression of $P_{R}$ only slightly, indicating that the $O_9$–$O_{R}$ loop was not disrupted.

These results show that, despite CI binding at $O_3^3$ being critical for repression of $P_{RM}$, it does not contribute strongly to the $O_9$–$O_{R}$ interaction, supporting the idea of a specific interaction between $O_3^3$ and $O_{R}$.

**Whole phage studies—the role of $O_3^3$ in CI autoregulation and prophage induction**

The function of $O_3^3$ in $\lambda$ 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 $O_3^3$ in CI repression of $P_{RM}$ now suggest a role for $O_3^3$, 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 $O_3^3$ actually facilitates prophage induction, the $O_3$-4 mutation was introduced into wild-type $\lambda$ to give $\lambda O_3^3$-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 $\lambda r1$ [carries $O_9^3$-$r1$ mutation] and $\lambda O_3$-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 $\lambda r1$ and $\lambda O_3$-4 lysogens, respectively. Thus, the $O_3$-4 and $O_3$-$r1$ mutations each caused a similar increase in the lysogenic CI concentration, confirming that $O_3^3$ is needed for CI repression of $P_{RM}$ in the native $\lambda$ 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 $O_3$-4 lysogen displayed a defect in prophage induction very similar to that of the $\lambda r1$ 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 $O_3$-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 $O_3$-4 mutation [data not shown].

Thus, our results provide an explanation for the retention of the third operator at $O_3$, indicating that the primary role of $O_3^3$ is to provide for CI negative auto-regulation in order to limit the CI concentration in the lysogenic state and allow efficient switching to lytic development.

**Localizing CI CTDs at $O_3$ is sufficient to facilitate repression of $P_{RM}$**

According to our model, the role of $O_3^3$ in $P_{RM}$ repression is to localize the CTDs of a dimer for contact with
the CTDs of a CI dimer bound at O₃ [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 O₃.

To test this prediction, we replaced O₃ on the chromosomal P₄ operator constructs with the P22 O₄[1] operator [Poteete et al. 1980]. λ operators are 17-bp long, and the center-to-center spacing of O₂ and O₃ is 20 bp; in the replacement, the spacing between O₂ 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 [λatt80] 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 O₄[1] operators of these phages are identical [Poteete et al. 1980].

Repression of P₄ was measured as P₄−/I− LacZ activity (= unpressed) divided by the activity of wt P₄. In the absence of the hybrid repressor and with wt O₃, P₄ was repressed 2.5-fold [Fig. 3, line 1]. As expected, replacement of λO₃ with the P22 operator prevented this repression of P₄ by λ repressor [Fig. 3, line 4]. [Note that in the absence of repression a P₄−/I− repression value of 1.1 is obtained because the r₁ mutation improves P₄ activity by about 10%; Dodd et al. 2001]. Because we expect the resident reporter-derived 21 repressor to occupy the O₃ position in this construct, this result indicates that repression of λP₄ is not enhanced by the binding of just any protein at the O₃ position. The presence of the hybrid repressor restored P₄ repression in the case of the reporter construct with the P22 operator at the position of O₃ [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 O₃ 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 O₃.

This experiment shows that repression of P₄ does not require a particular DNA sequence at O₃ or a particular DNA binding domain bound at O₃. Instead, effective repression can be achieved simply by tethering a pair of λ CI CTDs at O₃ for interaction with the CTDs of a CI dimer bound at O₃.

Figure 2 showed that repression of P₄ was lost when either O₃ or O₃ was mutated to prevent CI binding. The data in Figure 3 show that a similar loss of repression occurs when both O₃ and O₃ are mutated, indicating that the two mutations are not acting independently, but rather to disrupt the same repressive mechanism. The P₄ activity of the O₃-P₄ double mutant [857 units, Fig. 3, line 4] was similar to the activities seen with either single mutant [Fig. 3, line 1, O₃ P₄−/I− = 771 units; line 4, O₃-P₄−/I− = 798 units].

We wished to test the inference that interaction between CI dimers at bound at O₃ and O₃ 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
the P22-λ hybrid repressor. This substitution abolishes cooperation 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_3 and O_R3 is mediated by the same interface used for short-range dimer–dimer interactions.

The O_3-O_R3 interaction alone is insufficient for CI autoactivation

We hypothesized that the CI-mediated interaction between O_3 and O_R3 occurs efficiently only because the formation of a CI octamer linking O_R1-O_R2 and O_1-O_2 juxtaposes O_3 and O_R3 suitably for the binding of a CI tetramer. We tested this hypothesis by examining whether O_3 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_3 and O_R3 spaced at 5.8 kb [precisely the same distance as in the Fig. 2 constructs] but does not carry O_1, O_2, O_R1, or O_R2. There was no activation of P_RM in these reporters because O_R2 is absent. We found that O_3 versions of this reporter were only slightly repressed at 4 CI WLU (using pZC320cI, data not shown), so we introduced a higher range of CI concentrations (up to 60 WLU) by using a high-copy CI expression plasmid, pZE15cI [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_3 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_3 was also occupied. However, there was no enhancement of P_RM repression by the presence of an intact O_R3 at 3.8 kb [Fig. 4]. Thus, cooperativity at this distance between CI bound at O_3 and O_R3 requires the presence of the other λ operators.

### Physicochemical modeling of the CI-O_3-O_R3 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_3 and O_R3. 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

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**Table 1. Parameters used in physicochemical modeling**

| Parameter | Value | Promoter | LacZ units |
|-----------|-------|----------|------------|
| K_{dim}^a | 6.7 × 10^7 M^{-1} | P_R basal | 1056 |
| K_{NS}^{a} | 2.5 × 10^4 M^{-1} | P_R repressed | 2 |
| [NS]^b | 6.76 × 10^{-7} M | P_RM basal | 45 |
| [CI]_{hydrosyamic}^c | 3.7 × 10^{-7} M | P_RM activated | 360 (265) |
| | | | P_RM repressed | 0.5 |

^aO_R values from Kobl and Ackers [1992]; O_3 values from Seaver et al. [1986].
^bCalculated from ΔΔG values of Sarai and Takeda [1989]; see Dodd et al. [2001] for sequences.
^cSet as equal to nonspecific binding.
^dCI dimerization [Kobl and Ackers 1991].
^eAffinity 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.
^fConcentration 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].
^gCalculated from the data of Reichardt and Kaiser [1971] and 1 molecule/cell = 1.47 nM.
^hWild-type values; value in parentheses for looped configuration. P_R values for mutants [basal/repressed]: O_3-4 998/2, no O_1. 1164/2. P_RM values for mutants [basal/activated/repressed]: Δ12 37/240/180/0.5, r1 47/380/280/0.5.
$O_1-O_8$ 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 $\Delta G$ 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 $O_1-O_8$ interaction by also considering looped-unlooped equilibria, introducing nine looped configurations and two new cooperative free energy terms, $\Delta G_{\text{occ}}$ and $\Delta G_{\text{tet}}$. 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 $\Delta G_{\text{occ}}$ 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 $O_1-O_8$; together with the cost of formation of a DNA loop (Fig. 1B,C). The $\Delta G_{\text{tet}}$ cooperativity term was added only in the case where all six operators were occupied [applied in addition to $\Delta G_{\text{occ}}$]. 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, $\Delta G_{\text{tet}}$ 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 [deregressed 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 $\sim 10$-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 $K_{\text{occ}}$ (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 $O_1$.

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 $\sim 0.5$ kcal/mole for $\Delta G_{\text{occ}}$ and $\sim 3$ kcal/mole for $\Delta G_{\text{tet}}$. Figure 5A shows the simulations for $P_{RM}$ when $O_R$ is wild-type or carries mutations in $O_{R3}$ that abolish $\epsilon[1]$ or improve $\epsilon[12]$ 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 $O_1$ and also the very slight effect of the $O_13-4$ 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 $\Delta G_{\text{occ}}$ and $\Delta G_{\text{tet}}$, on the fit of the simulations with the data are shown for wild-type $P_{RM}$ in Figure 5C,D. With $\Delta G_{\text{tet}}$ held at $\sim 3$ kcal/mole, lowering $\Delta G_{\text{occ}}$ below $\sim 0.5$ kcal/mole produced a more sensitive CI activation of $P_{RM}$, whereas increasing $\Delta G_{\text{occ}}$ values above $\sim 1$ kcal/mole weakened CI repression of $P_{RM}$ (Fig. 5C). Setting $\Delta G_{\text{tet}}$ to $\sim 5$ 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 $\Delta G_{\text{tet}}$ while holding $\Delta G_{\text{occ}}$ at $\sim 0.5$ kcal/mole also increased CI repression of $P_{RM}$ (Fig. 5D).

Figure 5E shows the population distribution of the 64 different operator occupancy species [looped and unlooped configurations are combined] as a function of total repression concentration, calculated using $\Delta G_{\text{occ}} = 0.5$ and $\Delta G_{\text{tet}} = 3$ 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 $\Delta G_{\text{occ}}$ 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 R12L23 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 ($\Delta G_{\text{occ}} = +3$). 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 $O_1-O_8$ model

Our results support the model depicted in Figure 1 describing the CI interactions that regulate transcription from the $O_1-O_8$ region.
First, O$_3$ was shown to be necessary for CI repression of P$_RM$ at physiological CI concentrations. Blocking binding of λ CI to O$_3$, either by the O$_3$-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$_2$ was located 3.8 kb away from O$_R$. The requirement for O$_3$ in CI negative autoregulation and efficient prophage induction was confirmed in the native phage context where O$_3$ and O$_R$ are separated by 2.4 kb. Because O$_R$ 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$_3$ is unable to repress P$_RM$ directly [Fig. 4], we conclude that O$_3$ works by fostering CI binding at O$_R$.

Second, the proposal that O$_3$ facilitates CI binding at O$_R$ through a direct interaction between the dimers bound at O$_3$ and O$_R$ was supported by the finding that O$_3$ did not contribute significantly to the enhancement of CI repression of P$_RM$ by O$_3$, indicating that CI at O$_3$ does not interact significantly with CI bound to O$_R$ or O$_R$ [Fig. 2B]. The conclusion that CI bound at O$_3$ interacts primarily with CI bound at O$_R$ 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$_3$ and O$_R$ sites becoming favored at higher CI concentrations.

Third, the idea that the sole role of the O$_3$-bound CI dimer in facilitating P$_RM$ repression is to make a favorable protein–protein contact with the dimer bound at O$_R$ was supported by the demonstration that the CI dimer bound at O$_3$ could be functionally replaced by a hybrid repressor bearing the λ CI CTD but a heterologous DNA-binding domain. Specifically, O$_3$ 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$_3$ and O$_R$ did not cooperate in the absence of the other operators, even at CI concentrations where O$_3$ and, presumably, O$_R$ were occupied [Fig. 4]. Thus, the energetic benefit of the O$_3$-O$_3$ interaction must not outweigh the energetic cost of DNA looping. This supports the idea that the O$_R$-L$_1$.O$_R$-O$_1$.L$_1$ interaction, which mediates repression of P$_R$ and P$_E$ 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_{1,0.2} and O_{1,0.2} presumably juxtaposes O_{1,3} and O_{0.3} 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 $\Delta G_{oct} = -0.5$ kcal/mole for CI octamerization with a 3.8-kb DNA loop and an estimate of $\Delta G_{tec} = -3.0$ kcal/mole for the O_{0.3-0.3} 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 [$\Delta G_{oct}$ and $\Delta G_{tec}$] used in our physicochemical model cannot provide structural information. Formally, $\Delta G_{oct}$ represents a stabilization of the four-dimer CI complex and $\Delta G_{tec}$ 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_{1} and O_{0}. In theory, the four-dimer complex could be a pair of ‘trans’ tetramers, for example, a CI tetramer linking O_{1} and O_{0.1} with another tetramer linking O_{2} and O_{0.2}. 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_{1,3} and O_{0.3}, 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_{1,0.2} and at O_{0,1,0.2}? The $\Delta G_{oct}$ cooperativity term represents the net free energy change when the four-dimer complex goes from the unlooped to the looped configuration. $\Delta G_{oct}$ 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, $\Delta G_r$ and $\Delta G_{int}$. $\Delta G_r$ is a favorable change due largely to protein–protein association and is equal to $-RT \ln K_r$, where $K_r$ 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_{1} and O_{0}, when the two sites are unlinked. $\Delta G_r$ is an unfavorable change due to bringing the two DNA sites together. For a DNA loop of this size, $\Delta G_{int}$ 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 $\Delta G_r$ nor $\Delta G_{int}$ 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 $\Delta G_r$ [-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 (Se- near 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 $\Delta G_{int}$ can be obtained from modeling of in vitro DNA cyclization. Because $\Delta G_{int} = \Delta G_{oct} - \Delta G_r$, $\Delta G_{oct} = -RT \ln K_c$ and $\Delta G_r = -RT \ln K_r$ [see above], then $\Delta G_{int} = -RT \ln [K_r/K_c]$. The ratio $K_r/K_c$ 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 $1.1 \times 10^{-8}$ M [Eq. 3: Kuhn length $l = 100 \text{ nm}$, $L_m = 0.34 \text{ nm per bp}$, $d = 0$; Rippe 2001], giving an estimate of $\Delta G_{int} = 11.3 \text{ kcal/mole}$. Note that, by this calculation, the DNA tether makes the concentration of O_{1} relative to O_{0} sevenfold higher than the concentration of an unlinked O_{1} site in the cell [$\sim 1.5 \times 10^{-6}$ M; Table 1]. Combining these provisional estimates for $\Delta G_r$ and $\Delta G_{int}$ gives a $\Delta G_{oct}$ value of $+2.2 \text{ kcal/mole}$, considerably higher than our $\Delta G_{oct}$ value of $-0.5 \text{ kcal/mole}$ 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_{1}–O_{0} interaction in vivo be explained?

There are two reasons why $\Delta G_r$ might be more favorable than our provisional estimate of $-9.1 \text{ kcal/mole}$. 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_{1,0.2} and O_{0,1,0.2} 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_{1} and O_{0} 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 $\Delta G_r$.
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 $O_1$--$O_2$ 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 $\Delta G_{12}$ is DNA supercoiling. Supercoiling is expected to assist in vivo interactions between DNA sites because it should cause the DNA to wind into compact plektonemic 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 [Bartol 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 $O_2$ and $O_3$ on the same DNA molecule is not important in the CI octamerization reaction and that $O_1$ 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 $O_3$ is in part acting in this way, we believe that the ’tethering’ of $O_1$ to $O_2$ by the intervening DNA is critical. As discussed above, the linkage of $O_2$ to $O_3$ by 5.8 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 increase if $\Delta G_{12}$ 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 CI--$O_1$--$O_2$ 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 [ΔlacIZYA]74 galOP308 Str8 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 plasmid pUH1]. att80 was driven by 680att 680 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 kimm21 phage lacZ reporter vectors AR545ΔYA (deletion in lacY and lacA; no O1) and AR545ΔYA01 (carrying P1::O1, O2, O3, depicted in Fig. 2A) is described in Dodd et al. [2001]. AR545ΔYA01, O3, AR545ΔYA01, O3−4, and AR545ΔYA01, O3P22 were made using the same approach. AR545ΔYA01, O3−4 is the same as AR545ΔYA01 except that the sequence of O3 bears four changes [underlined] to inactivate CI binding: TATCATGAGTTCTTTAAT. In AR545ΔYA01, O3−4, O1 and O2 have been removed, leaving the sequence GAGATTTTAATAGTTCTTTAAT. In AR545ΔYA01, O3−4, O1−2 have been removed, leaving the sequence GGAATATTATCACCGCAGATGGTTAT (or its A1 derivative) between the BsrGI and SgrAI sites [O1, O2, and the remainder of O2 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 pPAM::lacZ and pPAM::lacZ operon fusions in pTL61T is described in Dodd et al. [2001]; the r1 version carries the O2–O1 mutation that inactivates CI binding. Construction of pPAM[Δ123-3]:lacZ and pPAM[Δ123-3]:lacZ operon fusions, in which O2 and O2 have been removed, was the same except that the cloned PCR product contained sequence from the −37 to +62 region of pPAM [4 bp of O2 are removed]. The pRwt::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 pZC320clI [mini-F origin, ApR, Pmin::clf] or pZC15clI [colEI origin, ApR, Pmin::clf], in combination with pUHA1 [supplies lac repressor], were used to supply IPTG-controlled levels of wt CI to lacZ reporters; cellular CI levels produced by pZE15 (with pUHA1) gave values of 2.5, 13.5, 38.9, 62.5, and 97.5 WLU (means of two extracts) for IPTG concentrations of 0, 50, 100, 200, and 500 µM, respectively.

The expression plasmid pFW7-280A [Whipple et al. 1998] was used to supply IPTG-controlled levels of the P22-α hybrid repressor, and plasmid pLR1cl [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 lacZ gene and provides resistance to chloramphenicol. Cells containing plasmids pFW7-280A [or pLR1cl] 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 $P_L$, 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].

$\lambda O_{3-4}$ construction and characterization
An $O_{3-4}$-containing PCR fragment containing $\lambda$ sequences from 35219 to 35858 was inserted into pBluescriptKS+ [Stratagene] to give pBS-$O_{3-4}$. The $O_{3-4}$ mutation [see above] was introduced into pBS-$O_{3-4}$ using the Quikchange method [Stratagene]. The mutant HpaI:35260-PvuI:35790 fragment was used to replace the equivalent wt fragment in pAP831, and the mutation was recombined in vivo from pAP831-$O_{3-4}$ onto lambda434 [Dodd et al. 2001], to give $\lambda O_{3-4}$.

Physicochemical modeling
The relative probability of each operator configuration, $s$, is given as

$$f_s = \exp(-\Delta G_s / RT) [Cl]_s$$

where $\Delta G_s$ is the sum of the contributions of all the free energies [see Table 1] for a given configuration $s$, [Cl] 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

$$[Cl]_{total, monomer} = 2[Cl] + \frac{[Cl]}{K_{dim}} \times (2K_{NS} [Cl]_i NS)$$

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 $P$ activity when $O_1$ and $O_2$ were unoccupied, repressed $P$ activity if either $O_1$ or $O_2$ were occupied, basal $P_{RM}$ activity when $O_2$ and $O_3$ were unoccupied, activated $P_{RM}$ activity when $O_2$ but not $O_3$ were occupied, and repressed $P_{RM}$ activity when $O_3$ was occupied. Basal promoter values were taken from reporter activity in the absence of CI; for $P_{RM}$ [wt, r1, and c12], values were from Dodd et al. [2001], and for $P_L$ [wt, $O_{3-4}$, and no $O_4$], 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 CI [Dodd et al. 2001]. The activated $P_{RM}$ values for the looped species were set 26% lower, in line with the effect of CI on the maximal activity of $P_{RM}$-r1 [Dodd et al. 2001]. The values used are given in Table 1.

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