Role of cis-acting sites in stimulation of the phage \( \lambda P_{RM} \) promoter by CI-mediated looping

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Running title: Stimulation of \( \lambda P_{RM} \) promoter by a distant UP element

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ABSTRACT

The lysogenic state of phage λ is maintained by the CI repressor. CI binds to three operators each in the $O_R$ and $O_L$ regions, which lie 2.4 kb apart. At moderate CI levels, the predominant binding pattern is two dimers of CI bound cooperatively at each regulatory region. The resulting tetramers can then interact, forming an octamer and a loop of the intervening DNA. CI is expressed from the $P_{RM}$ promoter, which lies in the $O_R$ region and is subject to multiple regulatory controls. Of these, the most recently discovered is stimulation by loop formation. In this work, we have investigated the mechanism by which looping stimulates $P_{RM}$. We find that two *cis*-acting sites lying in the $O_L$ region are involved. One site, an UP element, is required for stimulation. Based on the behavior of other promoters with UP elements located upstream of the -35 region, we suggest that a subunit of RNAP bound at $P_{RM}$ binds to the UP element located in the $O_L$ region. In addition, an IHF binding site lying adjacent to the UP element plays a less critical role, but is required for stimulation of the weak *prm240* allele. A loop with CI at the $O_L2$ and $O_L3$ operators does not stimulate $P_{RM}$, while one with CI only at $O_L2$ provides some stimulation. We discuss possible mechanisms for stimulation.
INTRODUCTION

The lysogenic state of phage λ is maintained by the CI repressor, which represses two early lytic promoters, thereby preventing initiation of the lytic pattern of gene expression. Expression of the host SOS response disrupts the lysogenic state by promoting proteolytic self-cleavage of CI, inactivating it and leading to expression of the lytic genes. Remarkably, this regulatory state is balanced in such a way that it is almost completely stable under normal growth conditions, and almost completely unstable in the face of a sustained SOS response. Hence, the circuitry acts as a switch.

A major part of this behavior results from the interplay of multiple controls that influence the expression of the cl gene. These controls operate on the PrM promoter, which is regulated at the level of transcription initiation by several different regulatory events. First, CI acts as a positive regulator of its own expression. When CI is bound to the Or2 operator (Fig. 1D), it stimulates PrM ~10-fold (1, 2). Second, occupancy of Or2 is a non-linear function of CI concentration, arising from two mechanistic features of CI. CI dimerizes rather weakly, so that the concentration of the dimer, the DNA-binding species, increases non-linearly. In addition, CI binds cooperatively to Or2 and Or1. Or1 is a strong binding site, while Or2 is a weak binding site; cooperativity results in the two sites largely filling up in parallel, and with a sigmoid binding curve.

The combination of non-linear binding to Or2 and positive autoregulation of PrM results in a transition from a low to a high level of PrM expression over a relatively narrow CI concentration range. This is a simple example of emergent behavior, and it was one of the earliest regulatory circuits to be analyzed at a mechanistic level (3, 4).
This behavior is largely responsible for the wide range in stability of the lysogenic state; below a given threshold of CI concentration, the protein is no longer expressed at a rate sufficient to counterbalance its depletion by proteolysis.

More recently, two additional levels of control of $P_{RM}$ expression have been recognized (5-8). Both these mechanisms involve a long-range interaction between molecules of CI bound at the $O_R$ region and CI bound at the $O_L$ region, some 2.4 kb distant (Fig. 1E and F). Two types of loops have been identified. Interaction between pairs of dimers at $O_L$ and at $O_R$ form an octamer (Fig. 1E). At higher CI concentrations, additional dimers bind to the remaining operators, forming a dodecamer (Fig. 1F). In the latter form, $P_{RM}$ is repressed, since the $O_R3$ site is occupied, resulting in negative autoregulation (5, 6). Intrinsically, $O_R3$ is a weak binding site, and its occupancy requires dodecamer formation. At the level of CI present in a $\lambda$ lysogen, this effect represses $P_{RM}$ about two-fold compared to the level of expression without dodecamer formation (9).

Formation of the octamer leads to another form of positive autoregulation. It provides a further increase of about 2-fold in $P_{RM}$ expression (7, 8). This effect is most readily observed when CI cannot bind to $O_R3$ or to $O_L3$ due to mutations in these sites. It has also been observed in vitro on supercoiled templates (10). In this work, we study the mechanism by which this stimulation occurs.

One possible inroad into analysis of this mechanism is provided by our recent finding (11) that a mutant allele of the $P_{RM}$ promoter, $prm240$, is stimulated by looping to a far greater extent than is the wild-type $P_{RM}$. When cells are grown in minimal medium, $prm240$ is stimulated about 5-fold by looping, as judged by the behavior of reporter genes. Hence, we have included analysis of $prm240$ in the present study.
It is plausible that looping-mediated stimulation operates, at least in part, with the involvement of cis-acting sites in the vicinity of the $O_L$ operators. We previously found (12) that the $T_{imm}$ terminator (Fig. 1A) and the 160 bp between $T_{imm}$ and $O_L$3 is highly conserved among $\lambda$ and a set of phages with $\lambda$ immunity specificity that were isolated from the wild. In $\lambda$, previous studies (13-16) show the existence of two sites adjacent to $O_L$3 (Fig. 1B). The first is a binding site for the host IHF protein, lying adjacent to and slightly overlapping $O_L$3. We term this the IHF site for brevity; it is sometimes termed L1 to distinguish it from another IHF binding site (not depicted), termed L2 and lying close to $T_{imm}$. The second site is an UP element lying further upstream and partially overlapping the IHF site. It is known that the C-terminal domain (CTD) of the $\alpha$ subunit of RNAP can interact with UP elements, increasing the strength of certain promoters (17), and the magnitude of this effect is quite variable. Both the IHF site and the UP element stimulate the lytic $P_L$ promoter (13-16), as judged by reporter assays.

Two molecular mechanisms have been proposed (7, 8) to account for looping-mediated stimulation of $P_{RM}$. It might result from interaction of RNA polymerase bound at $P_{RM}$ with the UP element at $O_L$. This model predicts that removal of the UP element would abolish the stimulatory effect. In this work we have tested this prediction. Alternatively, looping might somehow lead to conformational changes, either in CI or in RNAP, that make the promoter more active. Here we have used templates mutated in one or more $O_L$ operators to test whether other loops can also stimulate $P_{RM}$.

We find that the UP element plays an important role in looping-mediated stimulation. Recent evidence from a separate study (9) also supports this conclusion. In addition, we find that other looped forms do not confer stimulation, arguing that looping...
per se does not suffice to support the stimulatory effect. Finally, we have examined the role of the IHF site, and find that it is also important in stimulation, particularly for the mutant prm240 promoter.

**MATERIALS AND METHODS**

**Media, chemicals and reagents:** LB and M9 minimal media are as described (18). LB was supplemented with 0.2% glucose and 1 mM MgSO₄ (19); the carbon source for M9 was 0.2% glucose. Restriction enzymes and DNA ligase were from New England Biolabs or Fermentas Inc. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and XGal were from GoldBioTech. Ortho-nitrophenyl-β-galactoside and Polymyxin B (both used in assay of β-galactosidase) were from Sigma Chemical Co. Pfu Turbo DNA polymerase for site-directed mutagenesis (20) was from Stratagene; GoTaq Master Mix (Promega) for routine PCR was used as directed by the supplier.

**Bacterial, phage and plasmid strains:** Bacterial strains, with relevant genotype only given, were the following: JL2497 = N99 lacZΔM15/F' lacP' lacZΔM15::Tn9 (2), used as wild-type; JL6142 = JL2497 Δ(lacIPOZYA) F' (21); JL6994 = JL6142/pJWL615/pJWL486 (22); and JL6995 = JL6142/pJWL615/pA3B2 (22). Other bacterial strains are listed in Table S1 in Supplemental Material. Plasmids were pA3B2, a pACYC184 CmR derivative encoding a weak lacP::cl fusion (2, 23); pJWL486, a vector control for pA3B2 (2); and pJWL615, a derivative of the SpcR plasmid pGB2, carrying the lacP gene (2). Construction of phages carrying Prm::lacZ protein fusion reporter genes was as described (11), and is detailed in Supplemental Material. These phages are derivatives of the Simons vector λRS45 (24), modified as described (2); a generic structure of this vector is shown in Fig. 2A below. Regulatory elements downstream (2) and upstream (24) of the
lacZ gene were introduced as described. Reporter phages are listed in Table S3 in Supplemental Material. Single lysogens of reporter phages were made in strains JL6994 and JL6995; those in JL6994 are indicated in the figures as “no CI”. Construction of other phage and plasmid strains is described in Supplemental Material. The O₅₁-3 allele (this work) changes O₅₁ to TACCA[^ATG]GAGTTGATA, where the underlined bases are C, C and G respectively in O₅₁⁺; these positions are important for CI binding (25), so this allele should abolish specific binding to O₅₁. The O₅₃-4 allele (6) changes O₅₃ to TATCA[^CTG]CTAGTGGTT (underlined bases are C, G, C and A in WT, respectively) and blocks specific CI binding to O₅₃. The r₇ mutation changes O₆₃ to TATC[^C]ACA CGCAAGGGATA (underlined base is C in WT) and is proposed (5) to weaken CI binding to O₆₃ by about 2.9 kcal/mol, based on measurements in the context of O₆₁ (25).

**Methods:** General phage methodology, including measurement of burst sizes and lysogenization frequencies, phage-by-plasmid crosses, and prophage induction, was as described (19). The test for single lysogens (26) and assay for β-galactosidase activity (11) were as described.

**RESULTS**

**Rationale for experimental approach**

We initially sought to identify *cis*-acting sites that contribute to the increase in promoter activity conferred by looping, using a classical genetic selection and screen (see Supplemental Material). This approach did not yield the desired mutants. Accordingly, we turned to a reverse genetics approach, testing whether site-directed changes in either
the IHF binding site or the UP element (Fig. 2) affected the activity of $P_{RM}$, using the following reporter system (11).

This system (Fig. 2C) has a $P_{RM}::cI::lacZ$ protein fusion, termed $P_{RM}::lacZ$ for brevity, with the $O_L$ region lying downstream of the reporter gene. A control (Fig. 2B) has only the $T_{imm}$ terminator, which lies 160 bp upstream of $O_L$ in $\lambda$; the remaining constructs have $T_{imm}$, followed by $\lambda$ sequence to and through the $O_L$ region, with various alleles of several cis-acting sites. All these constructs make the same mRNA, terminated at $T_{imm}$, so that expression levels are directly comparable among various mutant derivatives (8). To test the response of these reporters to a range of CI concentrations, cells carried a plasmid with a $lacP::cI$ fusion. This plasmid provides some CI in the absence of IPTG; with increasing amounts of IPTG, cells make progressively more CI. A control strain with no CI lacked this plasmid. As previously noted (11), this system behaves differently in rich medium (LB) and minimal medium (M9), in that much more CI is made in M9 than in LB, both without IPTG and at a given IPTG level. Hence, the shapes of the dose-response curves differ, and these shapes cannot be compared between the two growth media.

In the absence of CI, the unstimulated $P_{RM}$ promoter activity leads to a very low level of expression (1). As the CI level increases, a mixture of different CI-bound template configurations is present, so that at a given CI concentration the observed activity reflects the sum of activities of the various species. In the $T_{imm}$ control, the expression level is dictated by events occurring locally in the $O_R$ region. At moderate CI levels, the predominant species is one with CI bound cooperatively to $O_R$1 and $O_R$2, giving the CI-stimulated level of $P_{RM}$-directed $lacZ$ activity. At higher CI concentrations,
CI would be expected to bind to OR3, repressing PRM, and lacZ expression would decline, but in our experiments the CI concentrations are not high enough to have this effect.

When looping is allowed by providing the OL region, additional species can form as CI levels increase. The first is an octamer, in which the predominant occupancy pattern is most likely CI bound to OR1, OR2, OL1 and OL2 (Fig. 1E). This is the species in which additional stimulation of PRM is conferred by looping. At higher CI levels, additional CI dimers bind to OR3 and OL3, forming the dodecamer; the resulting complex has little or no PRM activity (Fig. 1F). We can largely prevent dodecamer formation by blocking CI binding to OR3 and OL3, allowing us to examine the activation curve. At high CI levels, the remaining operators should be saturated with CI, and the expression curve should reach a plateau. However, since octamer formation is apparently only marginally favored, with an estimated ΔG of -0.5 kcal/mole (6), not all of the template molecules will be looped; hence the population is a mixture of unlooped and looped species, and the plateau level underestimates the activity of the looped octameric species.

We assessed the effects of changing the cis-acting sites. To remove the UP element, we deleted it by removing residues -86 to -104 relative to the PL start site, giving a deletion we term ΔUP (Fig. 2E). To change the IHF site without changing the relationship between the UP element and the OL operators, we introduced two point mutations at positions known from other studies (14) to weaken IHF binding and function, and a third mutation (G -72 to A) that should weaken a second weak promoter, termed P1,2, which lies upstream of the PL promoter (sometimes termed P1.1) and is more active in the absence of IHF (15). The result is a triple mutant termed IHFmut. Finally, we deleted both the IHF site and the UP element, removing residues -71 to -101, giving a
deletion we term Δ(71-101). Constructs also contained one of several alleles of the OR region, as indicated below.

Effects on activation

To analyze activation of P_{RM} in the absence of repression, we prevented specific CI binding to O_L3 by including the O_L3-4 allele (6), and weakened binding to O_R3 by including the r1 mutation in O_R3 (5). These changes largely prevent dodecamer formation. As CI levels increased, levels of lacZ expression reached a plateau, and in most cases declined only slightly as CI levels increased further.

Presence of the O_L region stimulated wild-type P_{RM} about 1.6-fold, as judged by comparing the O_L3-4 reporter with the T_{imm} control (Fig. 3A), as previously observed in vivo (11) and, with a different allele of P_{RM}, in vitro (10). Deletion of the UP element abolished this stimulatory effect (Fig. 3A). Mutation of the IHF site weakened but did not abolish the stimulatory effect. Removal of both sites by the Δ(71-101) deletion resulted in mild inhibition of lacZ expression. This result suggests that both sites contribute to activation of P_{RM} at least somewhat independently of one another. We conclude that stimulation of P_{RM} by looping requires the UP element, and that the IHF site plays a role in this process, but one less significant than the UP element.

We also infer that the reduced expression level seen in the Δ(71-101) deletion is evidence that this template was able to form looped complexes; otherwise it is difficult to explain why the expression level differs from the unlooped T_{imm} control. We assume, here and below, that all the mutant templates we analyze likewise can assume the looped form, including a few that show an expression level similar to the T_{imm} control. Based on this assumption, we discuss in several cases how changes in the template might affect the
activity of the looped form. In this case (Fig. 3A), the reduced level suggests that $P_{RM}$ is less active in the looped form than in the unlooped form.

We next tested the effects of the mutations on $lacZ$ expression directed by the weak $prm240$ promoter in M9 medium at 30° C (Fig. 3D). Under these conditions the relative effect of looping on $lacZ$ expression is greatest (11). We found, first, that both the IHF site and the UP element were required for stimulation of the mutant promoter by looping. Second, with the $\Delta UP$ allele, the observed level of expression was lower than seen in the $T_{imm}$ control. This lower level once again implies that a loop can form on this template, and that in this looped form the promoter is less active than in the absence of the loop. Third, the $\Delta(71-101)$ deletion gave a value slightly but reproducibly lower than the $\Delta UP$ template. Fourth, if we assume that the $IHFmut$ template can form a loop, then loop formation has no effect on the activity of $P_{RM}$; that is, the IHF site is essential for looping-mediated stimulation under these conditions. Finally, at high CI levels, there was significant repression of the template with wild-type UP element and IHF sites. We previously interpreted this result to indicate that cooperative interactions can stabilize a dodecamer in which some of the CI dimers are bound non-specifically (11).

We then tested whether the IHF site was also essential for looping-mediated stimulation of wild-type $P_{RM}$ in M9 at 30° C. As in LB, looping stimulated the $O_{3-4}$ reporter about 1.6-fold relative to the $T_{imm}$ control (Fig. 3B). A template with the $\Delta UP$ mutation displayed a slight inhibition relative to the $T_{imm}$ construct, and the $\Delta(71-101)$ construct showed a somewhat lower activity. The template with $IHFmut$ showed a reduced level of stimulation.
Finally, we analyzed the effect of changing the growth medium on expression from _prm240_ by growth in LB (Fig. 3C). As previously observed (11), the mutant promoter was considerably weaker in LB than in M9, and the degree of stimulation by looping was less in LB. As in M9, mutation of the IHF site markedly reduced the stimulatory effect. The contrast with its slight effect on the _P_{RM}^-_ promoter suggests that the IHF site plays a greater role with the weaker _prm240_ promoter. _ΔUP_ reduced the level of expression below that seen with the _T_{imm}^+_ control, and _Δ(71-101)_ caused an even greater reduction. With the IHF mutation, at low CI levels the expression was higher than in the _T_{imm}^+_ control. This suggests that presence of the _O_L_ operators increases the occupancy of _prm240_ by RNAP at low CI, but that in the looped form the promoter is not stimulated in the absence of the IHF site.

These templates contain the _O_{R3}r1_ mutation, which has a slight stimulatory effect on _P_{RM}^-_ (5, our data not shown). In other experiments (not shown), templates with _O_{R3}^+_ instead of _O_{R3}r1_ showed essentially the same behavior as their _r1_ counterparts; hence, the behavior described above does not reflect a peculiarity of the _r1_ mutation.

Taken together, these findings reveal unexpected complexity in the mechanism of looping-mediated stimulation. In most cases, the plateau expression levels from looped constructs differed from those of the _T_{imm}^+_ control. Interpreting this finding is complicated (see Discussion), but we infer that the activity of the looped form, or the proportion of looped species, is changed in the mutants from the wild-type case. For those cases in which _lacZ_ expression is the same as the control, we assume that the looped form can also occur, and we infer that in these cases _P_{RM}^-_ activity is unaffected by looping.
The detailed effects of mutations in the UP element and the IHF site on the activity of the looped form depend on the growth medium and on the identity of the \( P_{RM} \) allele. With wild-type \( P_{RM} \), the UP element is required for stimulation; hence our data support the proposal (7, 8) that the interaction of RNAP bound at \( P_{RM} \) with the UP element at \( O_L \) plays a part in looping-mediated stimulation of \( P_{RM} \). In rich medium, the looped form lacking the UP element had the same activity as the unlooped form, while in minimal medium its activity was somewhat reduced relative to that of the unlooped form. The larger \( \Delta(71-101) \) deletion further reduced the activity of the looped form. Mutation of the IHF site resulted in a level of activity that was intermediate between those of the unlooped form and the unmutated looped form. Since deletion of both sites had lower activity than the \( \Delta UP \) mutation alone, it is plausible that the IHF mutation is not acting indirectly by reducing the effectiveness of the UP element, but instead that the IHF site makes a small independent contribution to the mechanism of stimulation.

With the \( prm240 \) allele, the \( \Delta UP \) allele reduced expression below that of the control in both media, and the \( \Delta(71-101) \) had a somewhat lower level yet. The IHF mutation reduced expression to the same level as the control; we infer that the looped form has activity roughly equal to the unlooped form. Thus, the IHF site appears to play a more important role when \( P_{RM} \) is weakened by mutation.

Effects on repression

The pioneering work of Dodd et al (6) suggested that loop formation accompanying octamer formation is only marginally favored energetically. In contrast, once the loop has formed, dodecamer formation is much more strongly favored. It is plausible that the energetics of dodecamer formation might be altered in the absence of
the IHF site and/or the UP element. We addressed this issue by analyzing reporter
constructs that allowed us to assay repression as well as activation of $P_{RM}$.

We used sets of reporters similar to those above, except that they were wild-type
at $O_L^+3$ and $O_R^+3$, allowing dodecamer formation to occur, and tested wild-type $P_{RM}$ and
$prm240$ in both LB and M9 minimal medium (Fig. 4). In each case, $lacZ$ expression at
low CI concentrations resembled that seen with the templates mutated in $O_L^+3$ and $O_R^+3$
(see Fig. 3). Expression rose from a low level to a level resembling that at the plateau
seen when repression could not occur (Fig. 3). At higher CI levels, a substantial decline
in expression was seen, which was more severe in cells grown in M9, presumably
because the CI levels at a given IPTG concentration are higher (11).

We interpret this pattern as follows. First, at relatively low CI concentrations, we
suggest that the predominant form with templates that allow looping is the octamer,
because the highest level observed was similar to that seen in the templates that did not
allow repression. As discussed above, with different templates the octameric looped form
had different activities. Second, at higher CI concentrations, the repressed dodecameric
form became increasingly predominant, and the overall activity decreased.

These data provide little or no indication that $IHFmut$ or $\Delta UP$ affect the energetics
of dodecamer formation. For a given promoter and growth condition, the CI
concentration at which repression ensued was about the same with the $O_L^+3$ template as
with the mutant templates. If dodecamer formation were more favored, for instance, one
would expect repression to take place at substantially lower IPTG levels. There is a hint
of this in the case of $prm240$ in M9, but the effect is small at best.
Activity of other looped forms

In addition to the complex depicted in Fig. 1E, other looped forms are likely to exist. Since the $O_L$ and $O_R$ regions are 2.4 kb apart in the $\lambda$ genome, and DNA is flexible over this distance, the two regions can approach each other in two orientations, termed anti-parallel and parallel (Fig. 5A), creating different relationships between the operators at the two regions. Although the spatial relationship between the two duplexes remains unsettled (9, 27) (see Discussion), for simplicity we depict them here as pointing in the same direction.

Anderson and Yang (7) considered looped forms in which CI is bound to $O_R$1 and $O_R$2, but not to $O_R$3 (since CI bound at $O_R$3 would be expected to repress $P_{RM}$). These forms are shown in Fig. 5B; in each case, the $O_R$ region is shown at the bottom and in the same orientation. Forms 1, 2, 5, and 6 are anti-parallel and forms 3, 4, 7, and 8 are parallel. Form 1 juxtaposes the UP element and $P_{RM}$ in this representation, but that depends on the relative disposition of the two duplexes (see Discussion). The forms also differ in whether the CI tetramer at $O_L$ lies at $O_L$1 and $O_L$2, or at $O_L$2 and $O_L$3. Those in the right column are the same as those in the left column, but with a fifth dimer (hatched) bound to the remaining free $O_L$ site. Anderson and Yang (7) considered several models that varied according to which forms in addition to form 1 are active in transcription. We sought to test the activity of these forms by altering various sites in the $O_L$ region. It is not presently possible to control the orientation, limiting the scope of these tests. If the loop-mediated stimulation of $P_{RM}$ requires only the formation of the loop per se, stimulation should occur with CI bound only to $O_L$2 and $O_L$3, but not to $O_L$1 (7, 8), as in forms 5 and 7. We tested this possibility by mutating $O_L$1, creating the $O_L$1-3
allele (Fig. 6 legend), with three changes in positions in $O_{L1}$ important for CI binding (25). This should allow a possible $O_{L2}$-$O_{L3}$ configuration to form. Higher levels of CI will likely be required for tetramer formation than at $O_{L1}$ and $O_{L2}$, since $O_{L2}$ and $O_{L3}$ are weak binding sites (25). To prevent CI binding to $O_{R3}$, templates contained the $O_{R3} r1$ mutation. We also tested whether tetramer formation at $O_L$ was required for looping-mediated stimulation by using a template with mutations at both $O_{L1}$ and $O_{L3}$. Its response to varying CI levels might also indicate how much CI is needed to give occupancy of $O_{L2}$.

When wild-type $P_{RM}$ was assayed in LB (Fig. 6A), the effects of mutations in $O_L$ were somewhat surprising. When both $O_{L1}$ and $O_{L3}$ were mutated, the level of expression was unexpectedly higher than the $T_{imm}$ control at higher CI concentrations. This finding indicates that at least some occupancy of $O_{L2}$ occurred. It suggests that stimulation mediated by looping could occur when only $O_{L2}$ is occupied. Alternatively, it is possible that protein-protein interactions stabilized non-specific CI binding to the altered $O_{L1}$ site, maintaining some form of the octamer.

When only $O_{L1}$ was mutated, the $lacZ$ expression level was similar to that of the unlooped $T_{imm}$ control at low CI concentrations, and somewhat lower at high CI concentrations. The requirement for higher CI levels to give repression is consistent with the weak binding of CI to $O_{L2}$ and $O_{L3}$ (see above). This finding suggests that when both $O_{L2}$ and $O_{L3}$ are occupied, looping-mediated stimulation cannot occur; instead, the looped form has a lower level of expression than that of the unlooped form. Its residual activity cannot readily be assessed, since we do not know the fraction of templates that are looped (see Discussion). In addition, the fact that some looping-mediated stimulation
is observed for the $O_{L1}'O_{L3}'$ template suggests that inhibition of activity when only $O_{L1}$ is mutated is due to CI binding to $O_{L3}$ on that template.

A similar pattern was observed in M9, except that the template retaining only $O_{L2}$ was nearly as active as the $O_{L3}-4$ template (Fig. 6B). Again we infer that CI occupancy of $O_{L2}$ suffices to stimulate activity in the looped form, though it remains possible that cooperative interactions support non-specific CI binding at $O_{L1}$. With the $prm240$ mutant promoter, assayed both in LB and in M9, only a small amount of stimulation was seen with the $O_{L2}$-only template, and the $O_{L2} - O_{L3}$ template exhibited a more marked reduction in activity than did the corresponding $P_{RM}^+$ template (Figs. 6C and 6D).

The finding that some stimulation occurred with the $O_{L2}$-only template suggests that a wider variety of looped forms can exist than had been believed. Though it may be structurally implausible, perhaps an octamer could form with a single dimer at $O_{L2}$ and three dimers at $O_{R1}$, $O_{R2}$ and $O_{R3}$. Presumably this form would be repressed due to CI binding at $O_{R3}$. This model predicts that a template with $O_{R3}^+$ and only $O_{L2}^+$ at $O_{L}$ might give less expression than its $O_{R3}$ $r1$ counterpart. We repeated the above analysis on a set of templates with $O_{R3}^+$. Contrary to this prediction, the pattern of expression was essentially the same as seen with the $r1$ versions (data not shown). This argues against the possibility that an octamer of the type mentioned can form.

**Activity of template with CI bound to $O_{L3}$**

In looped form 2 (Fig. 5), Anderson and Yang (7) suggested that CI at $O_{L3}$ would repress $P_{RM}$ due to steric hindrance of RNAP binding at $P_{RM}$. To test this proposal, we compared expression on $O_{L3}^+$ and $O_{L3}-4$ templates. In addition, to allow for the possibility that bound IHF could sterically prevent CI from binding to $O_{L3}$ (see Error!)}
Reference source not found.1 in Supplemental Material), we compared $O_{L3}^+$ and $O_{L3-4}$ templates in the presence of $IHF_{mut}$. All templates also contained the $O_R3 r1$ allele to prevent dodecamer formation or independent CI binding to $O_R3$. If CI binding to $O_{L3}$ is repressive in the looped configuration, we expected that the level of expression would be reduced, relative to the unlooped $T_{imm}$ control, when CI is bound to $O_{L3}$. We found instead with both pairs of templates that the level of expression with the $O_{L3}^+$ template was intermediate between the $T_{imm}$ control and the corresponding $O_{L3-4}$ template (Fig. 7). Since the effect was about the same in both pairs of templates, we infer that IHF binding does not have a large effect on CI binding to $O_{L3}$.

We believe that CI levels in these experiments sufficed to allow CI binding to $O_{L3}$. Otherwise, the curves for the $O_{L3}^+$ and $O_{L3-4}$ templates would be the same. In addition, the affinities of CI for $O_{L2}$ and $O_{L3}$ are about the same in vitro (25), and our data with the $O_{L1-3}O_{L3-4}$ reporter (Fig. 6) showed that $O_{L2}$ was occupied in this concentration range. We conclude that binding of CI to $O_{L3}$ does not repress $P_{RM}$ (as long as $O_R3$ is unoccupied), and infer that the level of expression is reduced to some extent relative to that when $O_{L3}$ is free. Recent structural modeling (9) also suggests that CI binding to $O_{L3}$ prevents RNAP from contacting the UP element.

We concluded above (“Effects on Repression”) that mutating the IHF site had little or no effect on the energetics of dodecamer formation. If IHF bound very tightly to its site, one would expect that IHF binding would compete with CI binding to $O_{L3}$, and that blocking IHF binding would favor dodecamer formation. We infer that IHF binding to this site is relatively weak, again suggesting that CI can bind to $O_{L3}$ to some extent in the absence of dodecamer formation.
The finding that CI binding to OL3 is not repressive suggests that there is not a severe clash between CI at OL3 and RNAP at PRM in the looped form. Recent structural modeling (9) is consistent with this conclusion.

**Properties of phages carrying IHF or ΔUP mutations**

To analyze the effects of IHF and ΔUP mutations on regulation of CI in the intact phage, we crossed the mutations onto λ, using a newly-developed selection for phage recombinants carrying a plasmid-borne OL region (see Text and Fig. S2 in Supplemental Material). Both the IHFmut and ΔUP mutants were mildly defective in burst size and lysogenization frequency (see Supplemental Material). They formed stable lysogens, but these showed drastic defects in prophage induction (Fig. S3). Several lines of evidence (see Supplemental Material) suggest that the defect in prophage induction results, at least in part, from reduced expression of PL, probably through defects in expression of Int and/or Xis proteins. The mutants also showed a growth defect under particular conditions (Supplemental Material), which can also be explained by effects on PL expression. Hence, these mutations have pleiotropic effects on λ gene regulation, and we cannot readily analyze their effects on PRM expression in the context of the intact phage circuitry.

At the outset of Results, we mentioned an unsuccessful selection for mutants defective in looping-mediated stimulation. The properties of the IHFmut or ΔUP mutants would likely prevent their isolation by this approach (see Supplemental Material). At the same time, this negative result suggests that there do not exist other cis-acting sites in the OL region that are required for stimulation by looping but not for PL function.
Our evidence indicates that the stimulation of $P_{RM}$ by CI-mediated looping requires an UP element located near the CI binding sites in $O_L$ and is influenced by an adjacent IHF binding site. We interpret these data to indicate that stimulation requires a relatively specific configuration of the looped complex. We then discuss models for the role of the UP element.

**Evidence for a specific activated complex and requirement for UP element**

Two lines of evidence indicate that stimulation of CI-mediated activation occurs in a relatively specific form of the looped complex. First, a template lacking $O_L1$ did not support stimulation (Fig. 6); in addition, this and several other mutant templates gave lower activity than the unlooped $T_{imm}$ control in the presence of CI (Figs. 3, 4, and 6). We infer from the reduced activity that loops are forming on these templates, and conclude that loop formation *per se* is not sufficient for stimulation. Second, the carboxy-terminal domain (CTD) of the RNAP $\alpha$ subunit of RNAP is known to contact the UP element in other promoters (17); for this to occur in the present case, RNAP bound at $P_{RM}$ must lie within reach of the UP element in the $O_L$ region.

The $\alpha$-CTD is linked to the $\alpha$-NTD by a flexible tether; this allows the $\alpha$CTD to interact with UP elements with a certain amount of variation in the spatial relationship between the promoter and the UP element. Our evidence supports the suggestion of Anderson and Yang (8) that an $\alpha$CTD contacts the UP element in the $O_L$ region, and that this somehow stimulates CI-mediated activation of $P_{RM}$. Evidence from a separate study (9) also supports this conclusion.
In a proposed model for the DNA-bound octamer (27), the two DNA duplexes were held apart by ~100 Å and pointing in roughly the same direction. With this relative orientation of the DNA duplexes, the anti-parallel form (form 1 in Fig. 5B) juxtaposes the UP element and the $P_{RM}$ promoter, while in the parallel form (form 3) these elements are much farther apart, so that the $\alpha$CTD could probably not reach the UP element. It is known that the flexibility and reach of the linker attaching the $\alpha$CTD to the rest of RNAP is not unlimited (28). In studies of the $rrnB$ P1 promoter, which has a strong UP element lying upstream of the -35 region, moving the UP element relative to the -35 region reduced or abolished stimulation (28). Hence, if the duplexes pointed in the same direction, as suggested (27), our findings would favor a model in which only the anti-parallel configuration allows stimulation.

However, recent structural modeling (9) suggests a different relative orientation of the two duplexes. When flexibility is allowed for the DNA and for the hinge region of CI, this modeling suggests, first, that the DNA duplexes are bent and not pointing in the same direction, and second that the $\alpha$CTD can contact the UP element in both the anti-parallel and parallel configurations (forms 1 and 3 in Fig. 5). These authors concluded that both configurations allow stimulation. They also found experimentally that both orientations of the $O_L$ region (located 2.3 kb upstream of the promoter in this case) gave the same degree of stimulation, suggesting either that both orientations allow stimulation and/or that the flexibility of DNA over this distance allows both orientations to occur.

More detailed structural information will be needed to resolve this issue.
We suggest two non-exclusive mechanisms by which the UP element stimulates CI-mediated activation of \( P_{RM} \) in looped complexes. First, the \( \alpha \)CTD-UP element interaction may drive the equilibria among various species towards RNAP binding, thereby increasing the proportion of closed complexes at \( P_{RM} \). Second, the \( \alpha \)CTD-UP element interaction may lead to conformational changes in the complex which increase the activity of the promoter. We first recall the early steps in transcription initiation, then discuss and evaluate these models in turn.

Transcription initiation proceeds through formation of an open complex, in which the two DNA strands are separated prior to initiation. Its formation can be simplified to a two-step model:

\[
K_B \quad k_f
\]

\[
R + P \rightleftharpoons RP_c \rightarrow RP_o
\]

where R, P, RP\(_c\) and RP\(_o\) are free RNAP holoenzyme, free promoter, and closed and open complexes, respectively; \( K_B \) is the association constant for binding; and \( k_f \) is the rate constant for the overall isomerization reaction. Values for these parameters can be measured \textit{in vitro} by kinetic analysis of transcription initiation (29). Changes in either parameter can affect the rate of expression from a promoter (30). For most promoters, including \( P_{RM} \) (31, 32), formation of the open complex is rate-limiting, and open complexes usually initiate promptly, leading to promoter clearance and accessibility of the promoter to subsequent RNAP molecules. Accordingly, open complexes and later
initiating forms are a small fraction of the total templates, and we can ignore them in considering the distribution of species.

According to the first model for the effect of the UP element, the effective value of \( K_B \) is increased. The equilibria among the species that can form in our activation experiments (Fig. 3) are depicted in Fig. 8. At the CI levels used in these experiments (Fig. 3), \( P_{RM} \) activity reached a plateau value as CI concentrations increased, suggesting that operator occupancy by CI was complete. Even in the wild-type case (with wild-type \( O_{L3} \) and \( O_{R3} \)), these are the predominant tetrameric species, since the \( O_{L1}-O_{R2} \) occupancy pattern is energetically favored (33) and it is likely that the \( O_{L1}-O_{R2} \) pattern is also favored. Hence, at the moderate CI concentrations at which looping-mediated stimulation occurs, this framework should be applicable to the wild-type as well. The forms include those with RNAP bound (“B”) at \( P_{RM} \) and those free (“F”) of RNAP; those in the parallel (“P”) and antiparallel (“A”) looped configurations; and those that are unlooped (“U”). In addition, our data support the existence of stimulated forms (“S”) in which the \( \alpha \)CTD contacts the UP element.

Equilibrium constants for these interconversions are not presently known, but some information is available. The energy change upon looping, termed \( \Delta G_{oc} \) (6, 9), has a value of about \(-0.5\) kcal/mole, based on fitting reporter data at various CI levels to physicochemical models of the \( \lambda \) circuitry. This value reflects the equilibrium between UF and AF (or between UB and AB) (Fig. 8). At a value of \(-0.5\) kcal/mole, about 70% of the templates would be in the looped form.

The \textit{in vivo} equilibrium constant is also not known for the interconversion between UF and UB. For the unlooped molecule, \( K_B = \frac{[UB]}{[RNAP] \cdot [UF]} \). Although
an *in vitro* value for $K_B$ has been measured for unlooped $P_{RM}$ (31, 32), in general the value of $K_B$ depends markedly on the ionic conditions (34, 35), and it is unclear how to relate these to *in vivo* conditions. The *in vivo* concentration of free RNAP is also not known. Hence, the fraction of time that $P_{RM}$ is occupied by RNAP cannot be calculated.

If we make the reasonable assumption, however, that this fraction is less than half, a simple and straightforward mechanism follows for the action of the UP element in looping-mediated stimulation. If, as seems likely, the UP element – αCTD interaction stabilizes AS relative to AB, it would drive the equilibrium between UB and AB towards AB + AS. This would increase the fraction of templates on which RNAP is bound, thereby increasing the overall activity of the promoter. A quantitative analysis of this model, which we term the “$K_B$ model”, is presented in Supplemental Material. If PS is as active as AS, the same argument would apply to the parallel forms.

In this model, no other changes would need to occur in the activity of the promoter to account for the stimulatory effect. There is a limit on the degree to which $P_{RM}$ activity could be stimulated by this mechanism, since the fraction of templates in the closed complex cannot exceed 1.0. This model predicts that in an *in vitro* system (10) the apparent degree of stimulation would be decreased with increasing RNAP concentrations, and under conditions that favor RNAP binding to $P_{RM}$ (34, 35).

This model is also consistent with the greater degree of stimulation observed with the mutant *prm240* promoter (Fig. 3, ref. (11)). We have suggested (11) that *prm240* reduces the value of $K_B$, since it changes the same base in $P_{RM}$ as does *prm116*, which has this effect (32). If this is the case, the proposed mechanism would allow a greater degree
of stimulation, because the fraction of the UB form on the unlooped template would be substantially lower than in the WT case.

It is plausible that the degree to which the α-CTD – UP element interaction is favored would be different in certain cases due to steric effects. For instance, preventing IHF binding, or occupancy of OR3 by CI, might weaken this interaction, thereby reducing stimulation by this mechanism, as observed in these two cases (Fig. 3 and 6). In addition, the equilibrium between PS and PB might differ from that between AS and AB.

A second, non-exclusive model for the effect of the UP element is that conformational changes in the $P_{RM}$:CI:RNAP complex lead to an increase in promoter activity. Ample precedent exists for such a model. The interaction of an UP element with the αCTD can stimulate either $K_B$ or $k_f$; the two published studies, with different promoters, concluded that it probably acts at both steps (36, 37). In addition, the αCTD can contact DNA non-specifically upstream of the -35 region. At several promoters, this interaction somehow stimulates a step subsequent to RNAP binding (38, 39).

In the case of $P_{RM}$, several examples (with unlooped templates) are compatible with effects arising from subtle conformational changes. First, when CI can bind only to $O_R2$ but not to $O_R1$ or to $O_R3$, the degree of stimulation of $P_{RM}$ in vivo is only five-fold instead of ten-fold (1). Second, the RNAP $\sigma^{70}$ subunit contacts CI, and this contact increases $k_f$ (31, 32, 40) in vitro; when this contact is altered by a mutation in $\sigma^{70}$, the interaction with WT CI increases $K_B$ but not $k_f$ (41). Finally, certain $P_{RM}$ mutations appear to weaken cooperative CI binding between $O_R1$ and $O_R2$ in vitro, an effect interpreted as resulting from a conformational change in RNAP (42).
With the mutant prm240 promoter, we saw differences from $P_{RM}^+$ in its responses to changes in growth media and in the cis-acting sites in the $O_L$ region, notably with templates carrying only a functional $O_L2$ at $O_L$ or the $IHFmut$ allele. Possibly $prm240$ is stimulated, at least in part, by a mechanism different from that operating on $P_{RM}^-$. We speculate (see Supplemental Material) that a higher proportion of $prm240$ templates are in the anti-parallel form than in the wild-type case; such an effect could contribute to the greater degree of stimulation for $prm240$, if AS has higher activity than PS. Finally, conformational changes such as those just cited may also affect the properties of RNAP:CI interactions at the mutant promoter or in the interaction with the UP element; for instance, the interaction with the UP element may be more favorable with $prm240$ than with WT $P_{RM}$ (see also Supplemental Material and Fig. S5).

It was previously suggested (10) that looping might stimulate $P_{RM}$ expression simply by increasing the occupancy of $O_R2$. However, this model predicts that increasing the level of CI with the unlooped template would afford the same level of expression as with the looped one, contrary to our data and that of Lewis et al. (10).

**Effects of mutational changes on expression**

With many mutant templates, we saw decreases in the level of lacZ expression, and in some cases the activity was lower than that of the unlooped $T_{imm}$ control (Figs. 3, 4, 6 and 7). Decreases could in principle arise from several sources. These include changes in the value of $\Delta G_{oct}$, or in the activity of AS or the proportion of AS on templates allowing AS formation. If PS cannot form, or its formation is less favored than that of AS, or it has lower activity than AS, then an increase in the proportion of parallel forms would also reduce the overall activity. A combination of all these changes is also
possible. Our data do not permit us to infer which of these possible changes occur in the various mutants we analyzed. However, decreases below the level of the unlooped control imply that some of the looped species (Fig. 5) are repressed, partially or completely, relative to the unlooped control.

Role of IHF site

We found that the IHF binding site adjacent to OL3 plays a role in looping-mediated stimulation of PRM (Fig. 3). With wild-type PRM, mutating this site reduced the level of stimulation; with the prm240 promoter, stimulation was abolished, but the residual activity was somewhat greater than seen with the ΔUP template. In addition, the Δ(71-101) deletion, which removes both the UP element and the IHF binding site, decreased PRM activity below that of a template containing only the ΔUP deletion. This suggests that the IHF site is not simply enhancing the contribution of the UP element to looping-mediated stimulation of PRM.

IHF bends DNA by roughly 180° (43). Such a bend could place the UP element in a location that is more favorable for interaction with the αCTD; possibly in the absence of a bend the αCTD-UP element interaction is less effective. This model is not, however, consistent with the greater effect of the Δ(71-101) deletion.

In studies of looping-mediated stimulation in vitro (10), done in the absence of IHF, looping increased the activity of a mutant PRM allele by a factor of about 1.6. Our findings suggest that adding IHF might increase the degree of stimulation.

Closing remarks

An additional complexity of this system deserves further investigation. Although the loop is conventionally depicted as a simple structure (Figs. 1 and 8), this segment of
DNA is most likely supercoiled a large fraction of the time. Supercoiling causes DNA to form a plectonemic superhelix (Fig. 8B left), in which a segment of duplex DNA (represented by a single line) forms a loop with the two arms intertwined (44). The two arms can slide or “slither” rapidly with respect to one another (arrows in figure), rapidly juxtaposing a given site on one helix with many sites on the opposite helix, raising their effective local concentration perhaps 100-fold relative to that on relaxed DNA (44). This “slithering” should favor octamer formation (Fig. 8B right) and formation of the anti-parallel form. In the Supplemental Material, we consider these issues and the likely impact of transcription on superhelix formation and stability. We also discuss how these issues affect interpretation of the present work.

Clearly, much remains to be learned about this relatively simple system. It has long been appreciated that the OR region is densely packed with interdigitated sites (3, 4). Our findings, taken with those of others (13-16), indicate that the OL region is similarly dense in sites, and that these sites play multiple roles. Time will tell whether the complexity of the λ regulatory regions is exceptional, or is typical of biological systems and only appears exceptional because of the great depth in which λ has been analyzed.

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REFERENCES


FIGURE LEGENDS

Fig. 1. Maps of λ, functions of CI, and regulation of CI expression. A. Map of the immunity region of λ. B. Expanded map, to scale, of the Oₗ region, showing the location of the Oₗ₁, Oₗ₂ and Oₗ₃ sites, to which CI binds, and the location of the UP element and IHF binding site. Another IHF binding site, termed L2, is located close to the Timm site and not shown here. C. Expanded map of the Oₘ region, to scale, showing the location of the CI binding sites Oₘ₁, Oₘ₂ and Oₘ₃ and the location of the Pₚₘ and Pₚ promoters. 'cI' and cro' indicate the start of the cI and cro genes. D. Positive autoregulation of Pₚₘ by CI bound at Oₘ₂; CI binds cooperatively to Oₘ₁ and Oₘ₂. Both subunits of a dimer contact a subunit in the other dimer (27), as schematized. E, F. Positive and negative autoregulation of Pₚₘ by CI-mediated looping. In E, an octamer forms by interaction of tetramers at Oₗ and Oₘ, and Pₚₘ is further stimulated about 2-fold. In F, binding of two more CI dimers leads to dodecamer formation, and repression of Pₚₘ. The details of protein-protein interactions in looped forms are unclear; likely possibilities for favored forms are shown. Adapted from (11) with modifications.

Fig. 2: Structure of reporter constructs and location of mutations. A. Simplified map of the Simons vector used (24). These vectors are derivatives of phage λ with the immunity region of phage 21 (imm²¹). The lacZ reporter is located in a non-essential region to the left of the λ attachment site (attλ). B, C, D. Reporter constructs. Maps are not to scale; maps in C and D are aligned to the one in B. In each case, the mRNA is terminated at Timm, so that levels of expression are directly comparable (8). Some constructs contained the r1 allele in Oₘ₃, which weakens CI binding (see Text). The first
construct (B) is a control, which cannot loop; the second one (C) cannot confer negative autoregulation, particularly when the rl allele is present; the third (D) can carry out negative autoregulation. The $P_{RM}::cI::lacZ$ protein fusion joins the first 18 codons of $\lambda$ cI (hatched box) to codon 9 of lacZ (2), and is termed $P_{RM}::lacZ$ in the Text for brevity. E. Locations of the mutations used to alter the IHF site and/or the UP element are indicated; these were isolated both with $O_L3\cdot4$ and $O_L3^+$ alleles (see Text). Location of the IHF consensus site is underlined. The -72 G→A change is expected to weaken $P_L2$. The 17-bp $O_L3$ site starts with the “AA” bases shown at the right end of the sequence; hence it overlaps slightly with the IHF site. Adapted from (11) with modifications.

Fig. 3: Effects of IHFmut and ΔUP on activation of $P_{RM}^+$ and prm240. Reporters (Fig. 2) contained the $O_L3\cdot4$ allele and the $O_L3$ rl mutation to prevent negative autoregulation. Cells were grown in the indicated medium, and assayed as in Materials and Methods. The results of a typical experiment are presented. In each pair of panels, the same strains were grown either in LB at 37°C (panels A and C) or in M9 at 30°C (panels B and D). The $O_L$ alleles are indicated in the inset. A and B: $P_{RM}^+$. C and D: prm240. In each case, we used two strains, one lacking CI (“no CI”), and a second one with a plasmid bearing a weak lacP::cI fusion. This strain was grown without IPTG (“0”) or with the indicated IPTG concentration, affording a range of CI levels. As described previously (11), at 2 mM IPTG, CI levels in the latter strain in LB at 37°C and in M9 at 30°C were about equal to and 3 times, respectively, the level in a lysogen (11). Some CI is made in the absence of IPTG; in LB at 37°C, the CI levels in cells grown at 0.01 and 0.2 mM IPTG were roughly 0.1 and 0.5, respectively, times the lysogen level (11).
Fig. 4: Effects of IHFmut and ΔUP on repression of $P_{RM}^{+}$ and prm240. The experiment was as in Fig. 3, except that templates carried $O_L3^+$ and $O_R^+$ to allow repression at higher CI levels. A. $P_{RM}^{+}$, grown in LB at 37° C. B. $P_{RM}^{+}$, grown in M9 at 30° C. C. prm240, grown in LB at 37° C. D. prm240, grown in M9 at 30° C.

Fig. 5: Forms of looped complexes. A. Anti-parallel (left) and parallel (right) looped forms. The $O_R$ and $O_L$ regions approach one another in two different ways. The operators can also be juxtaposed in different registers (e.g., as in part C). Not to scale. The relative spatial orientation between the two duplexes is not known (see Text) but they are shown as pointing in the same direction for simplicity. B. Forms of looped complexes. The numbering scheme differs from that in (7). CI dimers are depicted as spheres. Forms in the right column are the same as those in the left column, but with a fifth dimer (hatched) bound to the remaining free $O_L$ site. Hatched dimers are not part of the octamer. It is not certain that CI binds cooperatively to $O_L2$ and to $O_L3$. These sites are separated by only 3 bp, rather than the 6- or 7-bp spacing separating $O_R2$ from $O_R3$ and $O_R1$ respectively, for which cooperativity has been extensively analyzed (33). With the CI repressor of phage HK022, reducing the spacing lowers the cooperativity parameter $\omega$ and changes the conformation of CI in the cooperative complex (45, 46). Footprinting data with an intact $\lambda$ O_L region (47) were interpreted as indicating cooperative binding, but this interpretation involved the assumption that the value of $\omega$ between $O_L1$ and $O_L2$ is the same as between $O_L2$ and $O_L3$.

Fig. 6: Effects of the $O_L1$ mutation on stimulation of $P_{RM}^{+}$ and prm240. As in Fig. 3, cells were grown in two growth media as indicated; growth in LB and M9 was at 37° C.
and 30° C, respectively. Templates carried various alleles in the $O_1$ operators, as indicated; all had the $r1$ allele in $O_R3$.

Fig. 7: **Effect of CI binding to $O_1$3 on activation of $P_{RM}^+$**. Cells were grown and assayed as in Figure 3, except that for each panel the averages of two experiments are presented. **A.** growth in LB at 37° C; **B.** growth in M9 at 30° C. All templates contained the $r1$ allele in $O_R3$.

Fig. 8: **Interconversions of looped complexes and effects of supercoiling. A.**

Interconversions among looped complexes. Only complexes with CI tetramers bound at $O_11$ and $O_12$, and at $O_R1$ and $O_R2$, are shown. For simplicity the later stages in open complex formation are not depicted, although clearly these would play a role. In form AB, the $\alpha$CTD of RNAP can bind to the UP element, giving AS; the chelate effect would likely favor AS in the $AB \leftrightarrow AS$ equilibrium. We include a stimulated parallel form, PS, based on recent structural modeling suggesting (9) that the $\alpha$-CTD:UP element interaction can also take place in the parallel form. This work (9) assumes that the equilibrium constant for the $PB \leftrightarrow PS$ interconversion is the same as for $AB \leftrightarrow AS$, but direct evidence for this is lacking. **B.** Left, idealized structure of a plectonemic superhelix. The line represents a DNA duplex. Negative supercoiling causes the duplex to wrap about itself in a right-handed superhelix, as shown. Helices can readily slide with respect to each other (44), as indicated by the arrows. Right, supercoiled form of the CI-mediated loop. Supercoiling favors the anti-parallel configuration, as shown. See Supplemental Material for further discussion.
A. Maps not to scale

```
lacZ  att^x  imm^21
```

B.

```
O_{R1}  O_{R2}  cl::lacZ  T_{im}
```

```
P_{Ry} or
prm240
```

```
mRNA
```

No CI-mediated looping

C.

```
r1  IHF  L2  UP  O_{L1}  O_{L2}
```

Supports looping-mediated positive autoregulation but little negative autoregulation

D.

```
IHF  L2  UP  O_{L3}  O_{L1}
```

Supports looping-mediated positive and negative autoregulation

E.

```
CCCTGCAAAAAATAATTCATATAAAAACATACAGATAA
```

```
IHFmut
```

```
\Delta UP
```

```
\Delta(71-101)
```
A. 

anti-parallel

\[ \begin{array}{c}
3 & 2 & 1 \\
\hline
3 & 2 & 1 \\
\end{array} \]

parallel

\[ \begin{array}{c}
1 & 2 & 3 \\
\hline
3 & 2 & 1 \\
\end{array} \]

not to scale

B. 

1

UP 3 2 1

\begin{array}{c}
\rightarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

2

UP 3 2 1

\begin{array}{c}
\rightarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

3

1 2 3 UP

\begin{array}{c}
\leftarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

4

1 2 3 UP

\begin{array}{c}
\leftarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

5

UP 3 2 1

\begin{array}{c}
\rightarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

6

UP 3 2 1

\begin{array}{c}
\rightarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

7

1 2 3 UP

\begin{array}{c}
\leftarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}

8

1 2 3 UP

\begin{array}{c}
\leftarrow \\
\hline
1 & 2 & 3 \\
\end{array}

\begin{array}{c}
P_{RM} 3 2 1 \\
\end{array}