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Role of *cis*-Acting Sites in Stimulation of the Phage λ P_{RM} Promoter by CI-Mediated Looping

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The lysogenic state of phage λ is maintained by the CI repressor. CI binds to three operators each in the right operator (O_R) and left operator (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 subjected 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 RNA polymerase (RNAP) bound at P_{RM} binds to the UP element located in the O_L region. In addition, adjacent to the UP element lies a binding site for integration host factor (IHF); this site 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.

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 *c*I gene. These controls operate on the P_{RM} 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 O_R2 site in the right operator (O_R) region (Fig. 1D), it stimulates P_{RM} ~10-fold (1, 2). Second, occupancy of O_R2 is a nonlinear 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 nonlinearly. In addition, CI binds cooperatively to O_R2 and O_R1. O_R1 is a strong binding site, while O_R2 is a weak binding site; cooperativity results in the two sites filling up largely in parallel and with a sigmoid binding curve.

The combination of nonlinear binding to O_R2 and positive autoregulation of P_{RM} results in a transition from a low to a high level of P_{RM} 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 left operator (O_L) region, which lie 2.4 kb apart (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 λ lysogen, this effect represses P_{RM} about 2-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 (WT) 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_L3 are highly

Received 27 November 2012 Accepted 21 May 2013
Published ahead of print 24 May 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.02148-12.

doi:10.1128/JB.02148-12

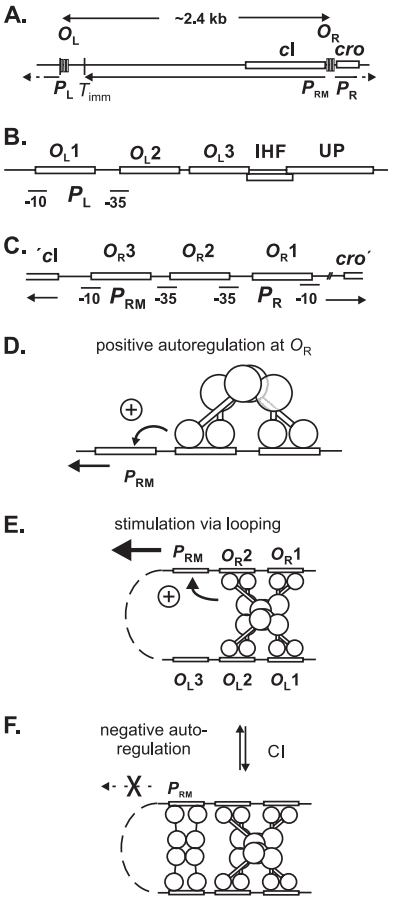

FIG 1 Maps of λ, functions of CI, and regulation of CI expression. (A) Map of the immunity region of λ. (B) Expanded map, to scale, of the O_L region, showing the location of the O_L1, O_L2, and O_L3 sites, to which CI binds, and the location of the UP element and IHF binding site. Another IHF binding site, termed L2, is located close to the T_{imm} site and not shown here. Binding of CI to O_L1 and/or O_L2 represses the lytic P_L promoter. (C) Expanded map of the O_R region, to scale, showing the location of the CI binding sites O_R1, O_R2, and O_R3 and the location of the P_{RM} and P_R promoters. " ′*c*I" and "*cro*′ " indicate the start of the *c*I and *cro* genes. Binding of CI to O_R1 and/or O_R2 represses the lytic P_R promoter. (D) Positive autoregulation of P_{RM} by CI bound at O_R2; CI binds cooperatively to O_R1 and O_R2. Both subunits of a dimer contact a subunit in the other dimer (27), as schematized. (E, F) Positive and negative autoregulation of P_{RM} by CI-mediated looping. In panel E, an octamer forms by interaction of tetramers at O_L and O_R, and P_{RM} is further stimulated about 2-fold. In panel F, binding of two more CI dimers leads to dodecamer formation and repression of P_{RM}. The details of protein-protein interactions in looped forms are unclear; likely possibilities for favored forms are shown. Adapted from reference 11 with modifications.

conserved among λ and a set of phages with λ immunity specificity that were isolated from the wild. In λ, previous studies (13–16) show the existence of two sites adjacent to O_L3 (Fig. 1B). The first is a binding site for the host protein integration host factor (IHF), lying adjacent to and slightly overlapping O_L3. 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 α subunit of RNA polymerase (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 previously (18). LB was supplemented with 0.2% glucose and 1 mM $MgSO_4$ (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 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were from GoldBio-Tech. 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 only relevant genotype given, were the following: JL2497, N99 *lacZ*ΔM15/F′ *lacI*^{q} *lacZ*ΔM15::Tn*9* (2), used as the 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 Cm^r derivative encoding a weak *lacP*::*c*I fusion (2, 23); pJWL486, a vector control for pA3B2 (2); and pJWL615, a derivative of the Spc^r plasmid pGB2, carrying the *lacI*^{q} gene (2). Construction of phages carrying P_{RM}::*lacZ* protein fusion reporter genes was as described previously (11) and is detailed in the supplemental material. These phages are derivatives of the Simons vector λRS45 (24), modified as described previously (2); a generic structure of these reporters is shown in Fig. 2A. Regulatory elements downstream (2) and upstream (24) of the *lacZ* gene were introduced as described. Reporter phages are listed in Table S3 in the 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 the supplemental material. The O_L1-3 allele (this work) changes O_L1 to TACCAATGGAGTTGATA, where the underlined bases are C, C, and G, respectively, in O_L1^+; these positions are important for CI binding (25), so this allele should abolish specific binding to O_L1. The O_L3-4 allele (6) changes O_L3 to TATCACTAGAGTTGGTT (underlined bases are C, G, C, and A in the WT, respectively) and blocks specific CI binding to O_L3 (6). The *r1* mutation changes O_R3 to TATCACACGCAAGGGATA (underlined base is C in the WT) and is proposed (5) to weaken CI binding to O_R3 by about 2.9 kcal/mol, based on measurements in the context of O_R1 (25).

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

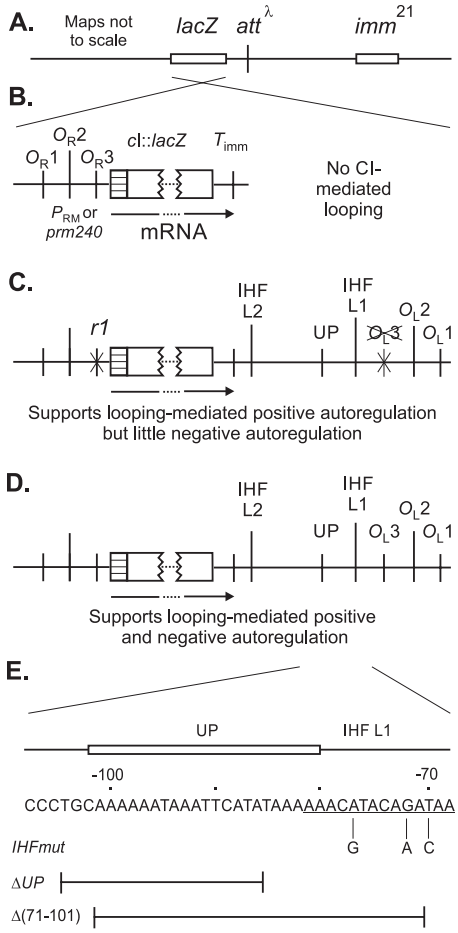

FIG 2 Structure of reporter constructs and location of mutations. (A) Simplified map of the reporter phages used, which are derived from a Simons vector (24). This vector is a derivative of phage λ with the immunity region of phage 21 (imm^{21}). The *lacZ* reporter is located in a nonessential region to the left of the λ attachment site (att^{λ}). (B, C, D) Reporter constructs. Maps are not to scale; maps in panels C and D are aligned to the one in panel B. In each case, the mRNA is terminated at T_{imm}, so that levels of expression are directly comparable (8). Some constructs contained the *r1* allele in O_R3, which weakens CI binding (see the text). The first construct (B) is a control, which cannot loop; the second one (C) cannot confer negative autoregulation, particularly when the *r1* allele is present; the third (D) can carry out negative autoregulation. The P_{RM}::*cI*::*lacZ* protein fusion joins the first 18 codons of λ *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_L3-4 and O_L^+ alleles (see the text). Location of a sequence resembling 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 reference 11 with modifications.

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 the 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_L3 in λ; the remaining constructs have T_{imm}, followed by λ 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_R1 and O_R2, giving the CI-stimulated level of P_{RM}-directed *lacZ* activity. At higher CI concentrations, CI is expected to bind to O_R3, repressing P_{RM}, 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 O_L 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 O_R1, O_R2, O_L1, and O_L2 (Fig. 1E). This is the species in which additional stimulation of P_{RM} is conferred by looping. At higher CI levels, additional CI dimers bind to O_R3 and O_L3, forming the dodecamer; the resulting complex has little or no P_{RM} activity (Fig. 1F). We can largely prevent dodecamer formation by blocking CI binding to O_R3 and O_L3, 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 change in free energy, ΔG, of −0.5 kcal/mol (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 P_L start site, giving a deletion we term Δ*UP* (Fig. 2E). To change the IHF site without changing the relationship between the UP element and the O_L operators, we introduced two point mutations at positions known from other studies (14) to weaken IHF binding and function, as well as a third mutation (G−72A) that should weaken a second weak promoter, termed P_L2, which lies upstream of the P_L promoter (sometimes termed P_L1) 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 O_R region, as indicated below.

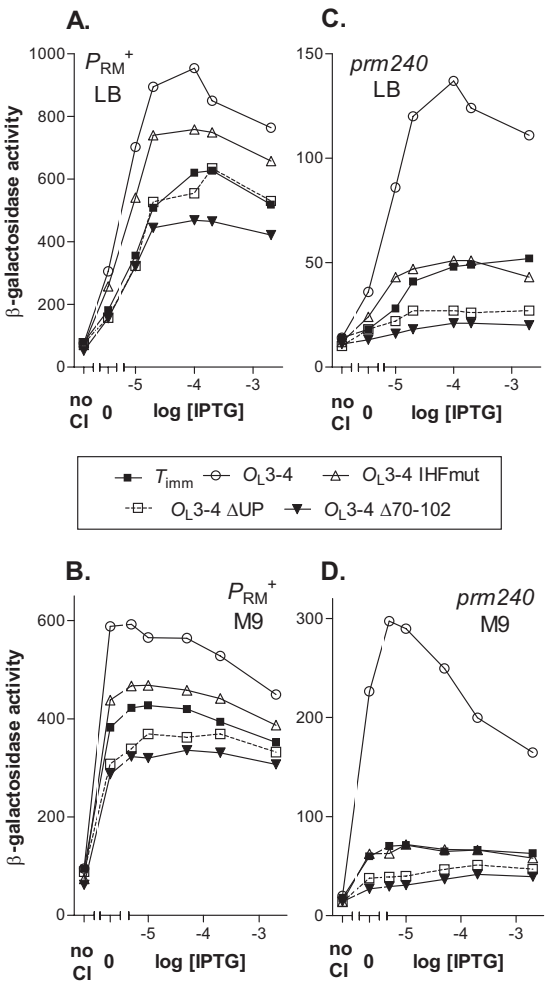

FIG 3 Effects of *IHFmut* and Δ*UP* mutations on activation of P_{RM}^+ and *prm240*. Reporters (Fig. 2) contained the O_L3-4 allele and the O_R3 *r1* mutation to prevent negative autoregulation. Cells were grown in the indicated medium and assayed as described in Materials and Methods. The results of a typical experiment are presented. In each pair of panels, the same strains were grown either in LB at 37°C (A and C) or in M9 at 30°C (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 times the lysogen level, respectively (11).

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 that of 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 Δ*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 Δ(71-101) deletion gave a value slightly but reproducibly lower than the Δ*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 the 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 nonspecifically (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_L3-4 reporter about 1.6-fold relative to the T_{imm} control (Fig. 3B). A template with the Δ*UP* mutation displayed a slight inhibition relative to the T_{imm} construct, and the Δ(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 from M9 to LB on expression of *prm240* (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. The Δ*UP* mutation reduced the level of expression below that seen with the T_{imm} control, and the Δ(71-101) deletion 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 deletion, Δ(71-101), 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 Δ*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 Δ*UP* allele reduced expression below that of the control in both media, and the Δ(71-101) deletion had a somewhat lower level. The IHF mutation reduced expression to the same level as the control; we infer that the looped form has activity roughly equal to that of 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 described above, except that they were WT at O_L3 and O_R3, allowing dodecamer formation to occur, and tested WT 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_L3 and O_R3 (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).

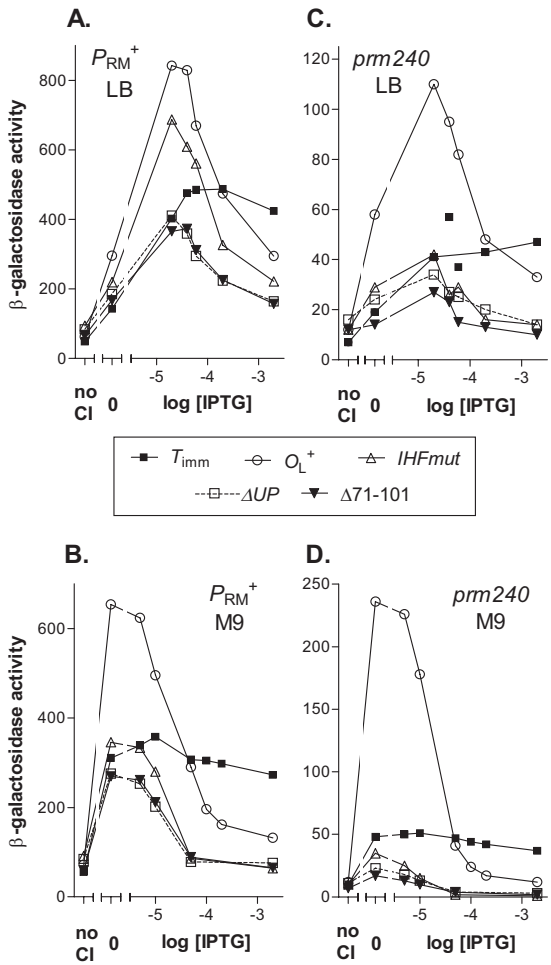

FIG 4 Effects of *IHFmut* and Δ*UP* mutations on repression of P_{RM}^+ and *prm240*. The experiment was as described for Fig. 3, except that templates carried O_L3^+ and O_R^+ to allow repression at higher CI levels. (A) P_{RM}^+, grown in LB at 37°C; (B) P_{RM}^+, grown in M9 at 30°C; (C) *prm240*, grown in LB at 37°C; (D) *prm240*, grown in M9 at 30°C.

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 the *IHFmut* or Δ*UP* mutations 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^+ 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 λ genome, and DNA is flexible over this distance, the two regions can approach each

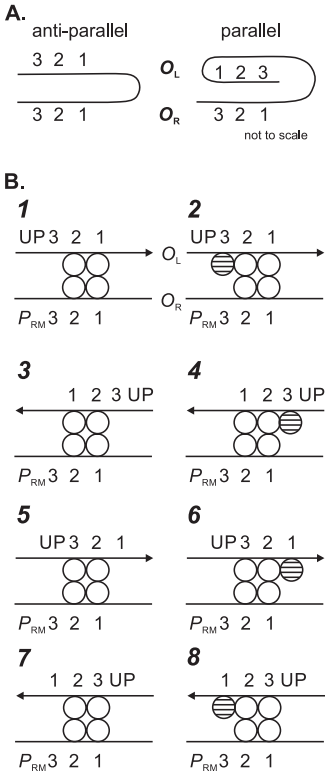

FIG 5 Forms of looped complexes. (A) Antiparallel (left) and parallel (right) looped forms. The O_R and O_L regions approach one another in two different ways. The operators can also be juxtaposed in different registers (e.g., as in panel B). Not to scale. The relative spatial orientation between the two duplexes is not known (see the 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 reference 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 ω and changes the conformation of CI in the cooperative complex (45, 46). Footprinting data with an intact λ O_L region (47) were interpreted as indicating cooperative binding, but this interpretation involved the assumption that the value of ω between O_L1 and O_L2 is the same as between O_L2 and O_L3.

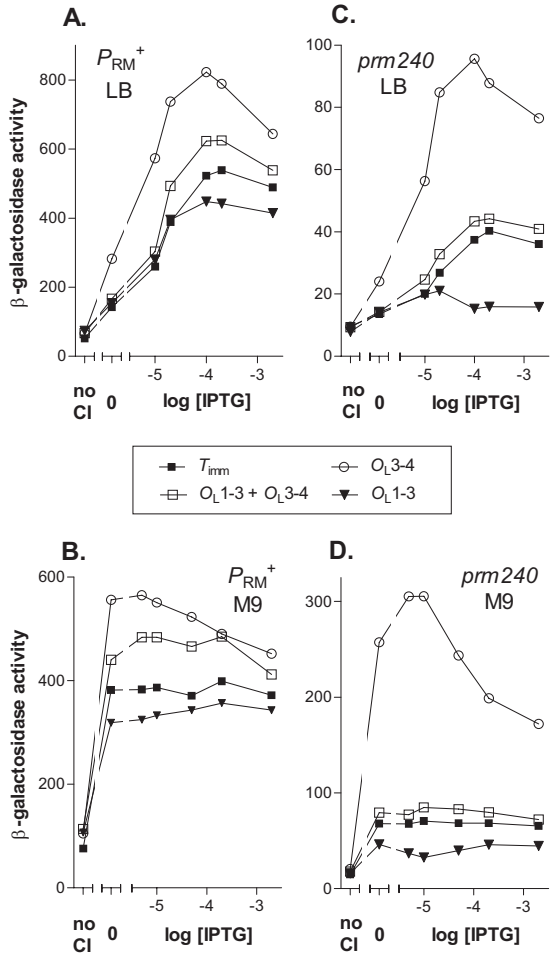

FIG 6 Effects of the O_L1 mutation on stimulation of P_{RM}^+ and *prm240*. As described for Fig. 3, cells were grown in two growth media as indicated; growth in LB and M9 was at 37°C and 30°C, respectively. Templates carried various alleles in the O_L operators, as indicated; all had the *r1* allele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

other in two orientations, termed antiparallel 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_R1 and O_R2 but not to O_R3 (since CI bound at O_R3 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 antiparallel 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_L1 and O_L2 or at O_L2 and O_L3. 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_L2 and O_L3 but not to O_L1 (7, 8), as in forms 5 and 7. We tested this possibility by mutating O_L1, creating the O_L1-3 allele (see Materials and Methods), 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 various 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 that of 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 nonspecific 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 nonspecific 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_L2-3 template exhibited a more marked reduction in activity than did the corresponding P_{RM}^+ template (Fig. 6C and D).

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 analysis described above on a set of templates with O_R3^+. Contrary to this prediction, the pattern of expression was essentially the same as that 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 Fig. S1 in the supplemental material), we compared O_L3^+ and O_L3-4 templates in the presence of the *IHFmut* mutation. 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 those of 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.

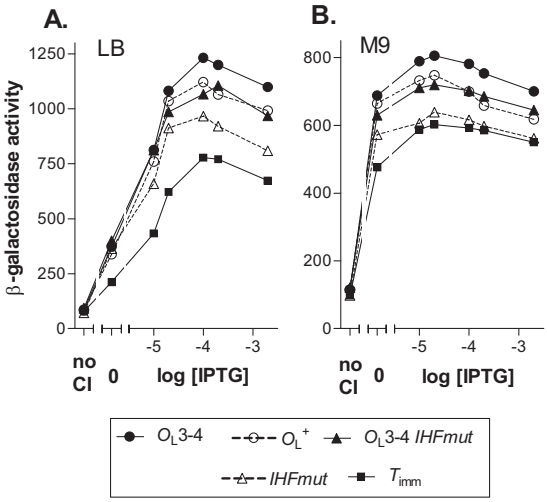

FIG 7 Effect of CI binding to O_L3 on activation of P_{RM}^+. Cells were grown and assayed as described for Fig. 3, except that for each panel the averages of two experiments are presented. (A) Growth in LB at 37°C; (B) growth in M9 at 30°C. All templates contained the *r1* allele in O_R3.

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 O_L3 is not repressive suggests that there is not a severe clash between CI at O_L3 and RNAP at P_{RM} in the looped form. Recent structural modeling (9) is consistent with this conclusion.

Properties of phages carrying IHF or Δ*UP* mutations. To analyze the effects of IHF and Δ*UP* 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 O_L region (see the text and Fig. S2 in the supplemental material). Both the *IHFmut* and Δ*UP* mutants were mildly defective in burst size and lysogenization frequency (see the supplemental material). They formed stable lysogens, but these showed drastic defects in prophage induction (see Fig. S3 in the supplemental material). Several lines of evidence (see the supplemental material) suggest that the defect in prophage induction results, at least in part, from reduced expression of P_L, probably through

defects in expression of Int and/or Xis proteins. The mutants also showed a growth defect under particular conditions (see the supplemental material), which can also be explained by effects on P_L expression. Hence, these mutations have pleiotropic effects on λ gene regulation, and we cannot readily analyze their effects on P_{RM} 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 Δ*UP* mutants would likely prevent their isolation by this approach (see the supplemental material). At the same time, this negative result suggests that there do not exist other *cis*-acting sites in the O_L region that are required for stimulation by looping but not for P_L function.

DISCUSSION

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 (Fig. 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 α 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 αCTD is linked to the α N-terminal domain (αNTD) by a flexible tether; this allows the αCTD 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 αCTD 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 pointed in roughly the same direction. With this relative orientation of the DNA duplexes, the antiparallel 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 the αCTD could probably not reach the UP element. It is known that the flexibility and reach of the linker attaching the αCTD to the rest of RNAP are 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 antiparallel 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 αCTD can contact the UP element in both the antiparallel 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.

Mechanism for stimulation by the UP element. We suggest two nonexclusive mechanisms by which the UP element stimulates CI-mediated activation of P_{RM} in looped complexes. First, the αCTD-UP element interaction may drive the equilibria among various species toward RNAP binding, thereby increasing the proportion of closed complexes at P_{RM}. Second, the αCTD-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 and 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:

$$R + P \overset{K_B}{\rightleftharpoons} RP_c \xrightarrow{k_f} RP_o$$

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 *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_R1 - O_R2 occupancy pattern is energetically favored (33) and it is likely that the O_L1 - O_L2 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 αCTD contacts the UP element.

Equilibrium constants for these interconversions are not presently known, but some information is available. The energy change upon looping, termed ΔG_{oct} (6, 9), has a value of about

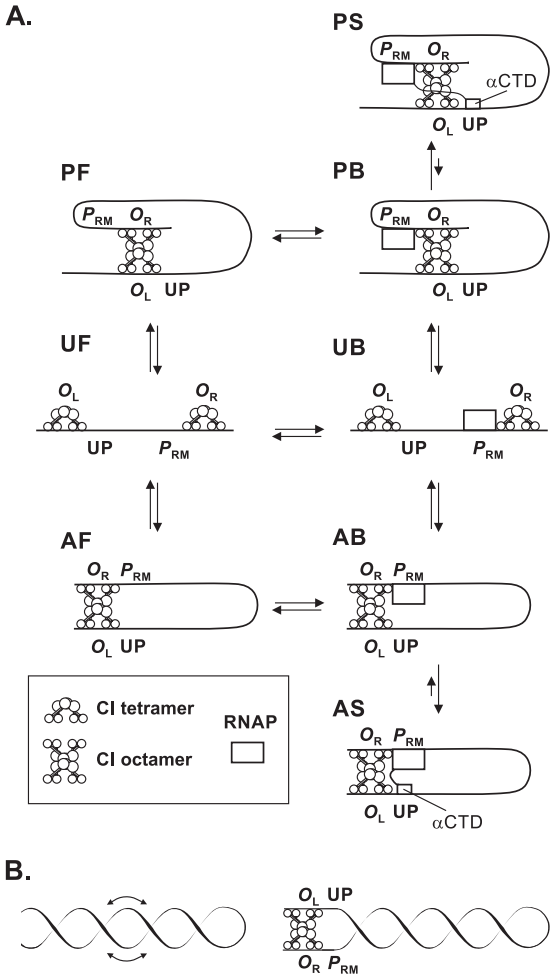

FIG 8 Interconversions of looped complexes and effects of supercoiling. (A) Interconversions among looped complexes. Only complexes with CI tetramers bound at O_L1 and O_L2 and at O_R1 and O_R2 are shown. For simplicity, the later stages in open complex formation are not depicted, although clearly these would play a role. In form AB, the αCTD of RNAP can bind to the UP element, giving AS; the chelate effect would likely favor AS in the AB ↔ AS equilibrium. We include a stimulated parallel form, PS, based on recent structural modeling suggesting (9) that the αCTD-UP element interaction can also take place in the parallel form. This work (9) assumes that the equilibrium constant for the PB ↔ PS interconversion is the same as for AB ↔ 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 antiparallel configuration, as shown. See the supplemental material for further discussion.

−0.5 kcal/mol, based on fitting reporter data at various CI levels to physicochemical models of the λ circuitry. This value reflects the equilibrium between UF and AF (or between UB and AB) (Fig. 8). At a value of −0.5 kcal/mol, about 70% of the templates would be in the looped form.

The *in vivo* equilibrium constant is also not known for the interconversion between UF and UB. For the unlooped molecule, K_B = [UB]/[RNAP] × [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 these coupled equilibria toward 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 the 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) (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 αCTD-UP element interaction is favored would be different in certain cases due to steric effects. For instance, preventing IHF binding, or occupancy of O_L3 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, nonexclusive 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 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 nonspecifically 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 5-fold instead of 10-fold (1). Second, the RNAP σ^{70} subunit contacts CI, and this contact increases k_f (31, 32, 40) *in vitro*; when this contact is altered by a mutation in σ^{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 the supplemental material) that a higher proportion of *prm240* templates are in the antiparallel form than in the wild-type case; such an effect could contribute to the greater degree of stimulation observed with *prm240* than with P_{RM}^{+}, 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 the text and Fig. S5 in the supplemental material).

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 those 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 (Fig. 3, 4, 6, and 7). Decreases could in principle arise from several sources. These include changes in the value of ΔG_{oct}, or in the activity of AS, or in 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 O_L3 plays a role in looping-mediated stimulation of P_{RM} (Fig. 3). With wild-type P_{RM}, mutating this site reduced the level of stimulation; with the *prm240* promoter, stimulation was abolished, but the residual activity was somewhat greater than that seen with the Δ*UP* template. In addition, the Δ(71-101) deletion, which removes both the UP element and the IHF binding site, decreased P_{RM} activity below that of a template containing only the Δ*UP* deletion. This suggests that the IHF site is not simply enhancing the contribution of the UP element to looping-mediated stimulation of P_{RM}.

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 P_{RM} 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 (Fig. 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 antiparallel 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 O_R region is densely packed with interdigitated sites (3, 4). Our findings, taken with those of others (13–16), indicate that the O_L 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.

ACKNOWLEDGMENTS

We are grateful to Gary Gussin for helpful discussions, to Sankar Adhya, Carol Dieckmann, and Gary Gussin for comments on the manuscript, to Anca Segall for plasmids, and to Ian Dodd and Keith Shearwin for communicating unpublished results.

This work was supported by grant GM24178 from the National Institutes of Health.

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