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Mutually Exclusive Utilization of P_R and P_{RM} Promoters in Bacteriophage 434 O_R

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Establishment and maintenance of a lysogen of the lambdoid bacteriophage 434 require that the 434 repressor both activate transcription from the P_{RM} promoter and repress transcription from the divergent P_R promoter. Several lines of evidence indicate that the 434 repressor activates initiation of P_{RM} transcription by occupying a binding site adjacent to the P_{RM} promoter and directly contacting RNA polymerase. The overlapping architecture of the P_{RM} and P_R promoters suggests that an RNA polymerase bound at P_R may repress P_{RM} transcription initiation. Hence, part of the stimulatory effect of the 434 repressor may be relief of interference between RNA polymerase binding to the P_{RM} promoter and to the P_R promoter. Consistent with this proposal, we show that the repressor cannot activate P_{RM} transcription if RNA polymerase binds at P_R prior to addition of the 434 repressor. However, unlike the findings with the related λ phage, formation of RNA polymerase promoter complexes at P_{RM} and at P_R apparently are mutually exclusive. We find that the RNA polymerase-mediated inhibition of repressor-stimulated P_{RM} transcription requires the presence of an open complex at P_R. Taken together, these results indicate that establishment of an open complex at P_R directly prevents formation of an RNA polymerase-P_{RM} complex.

Each lambdoid bacteriophage contains a right operator (O_R) region on its chromosome that is at the center of a complex regulatory circuit responsible for governing the phage's choice between lytic and lysogenic development. Proper regulation of transcription initiation from the divergently oriented P_R and P_{RM} promoters that lie within the O_R region is crucial to the lysis-lysogeny decision. In each phage, the activities of these promoters are regulated, in part, by the binding of the bacteriophage repressor to three recognition sites that partially overlap the P_R and P_{RM} promoters. In the absence of the repressor, the P_{RM} promoter is virtually inactive and RNA polymerase preferentially initiates transcription at the P_R promoter. During an infection or induction of a lysogen, continued activity of the P_R promoter drives the phage to develop lytically. If the phage is to develop or maintain the lysogenic state, there must be exclusive expression of P_{RM} over P_R.

To perform its role in the lysis-lysogeny decision, the repressor must bind to each of the three binding sites or operators within O_R with different affinities and act both as transcriptional activator of P_{RM} and as repressor of P_R. In a developing or existing lysogen, the repressor binds with highest affinity to two sites, O_R1 and O_R2. In this configuration, the repressor molecule bound at O_R2 activates transcription from the P_{RM} promoter. This event leads to expression of the *c*I gene that encodes the repressor, which is the sole protein responsible for maintenance of the lysogenic state. This binding configuration also permits the repressor to concurrently inhibit transcription initiation from P_R and in doing so prevents the transcription of genes needed for lytic growth (for a review, see reference 23).

Comparisons among the lambdoid phages have added to our understanding of O_R function (1) as well as provided insight into the general mechanisms of transcriptional regulation by DNA binding proteins. In recent years, much has been learned about how the repressors of these phages activate transcription. Evidence suggests that the O_R2-bound repressor of each phage activates transcription initiation by directly contacting the σ^{70} subunit of the P_{RM}-bound RNA polymerase (16, 18). Studies of bacteriophage λ indicate that, in addition to activating P_{RM} transcription by directly contacting RNA polymerase, the λ repressor also activates transcription indirectly by relieving interference with an RNA polymerase bound at P_R (6, 10, 11). Interestingly, in λ phage, formation of an open complex at the P_R promoter does not prevent RNA polymerase from binding at P_{RM} but rather impairs isomerization of the RNA polymerase-P_{RM} closed complex to an open complex (6, 10). These findings lead to the suggestion that formation of an open complex at the λ P_R promoter interferes with formation of a similar complex at P_{RM}. Consistent with this suggestion, on templates bearing a single base deletion in λ O_R, which decreases the distance between the transcription start site of P_{RM} and that of P_R, open complex formation at P_{RM} is drastically inhibited by RNA polymerase bound at P_R (26).

In the O_R region of bacteriophage 434, the transcription start sites of the P_R and P_{RM} promoters are separated by 65 bp, compared to the 82-bp separation found in λ's O_R region. This separation results in a strikingly different placement of the P_R and P_{RM} promoter elements with respect to the O_R2 sites in 434 phage relative to phage λ. In λ phage, the −35 elements of its P_{RM} and P_R promoters overlap the left and right ends of O_R2, respectively. In 434 phage, the −35 elements of 434 P_R and P_{RM} promoters are located on the left side of O_R2 and almost completely overlap (Fig. 1). As a result of this geometry, severe promoter interference between P_R and P_{RM} in 434 O_R was anticipated (2, 3). Until now, however, this prediction had not been confirmed.

The potential for simultaneous occupancy of P_R by RNA polymerase and the repressor at O_R leads to a question about the precise mechanism that the repressor uses in inhibiting transcription initiation from P_R. Jacob and Monod (13) advanced the idea that gene regulation could occur by preventing or repressing the expression of genes. Their classical model of repressor function proposes that repressors block access of

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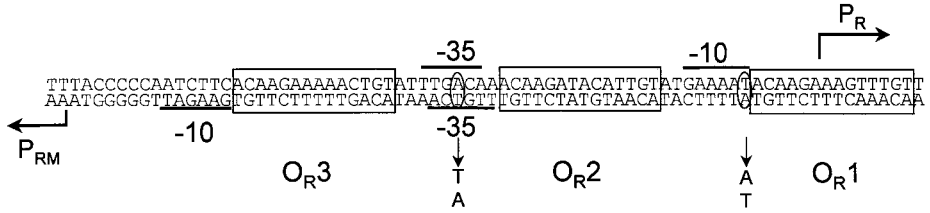

FIG. 1. The sequence of 434 O_R region. O_R1, O_R2, and O_R3 are enclosed in boxes; the transcription start sites of P_{RM} and P_R are indicated by bent arrows. The −35 and −10 regions of P_R and P_{RM} are underlined. The positions and sequences of the promoter mutants used in this study are indicated.

RNA polymerase to the promoter by occluding the RNA polymerase binding site. More recent studies indicate, however, that repressors of transcription often act subsequent to the formation of the initial RNA polymerase-promoter complex (4, 9, 17, 20).

MATERIALS AND METHODS

Enzyme and reagents. Wild-type and mutant 434 repressors were prepared as described in reference 24. Sigma-saturated wild-type *Escherichia coli* RNA polymerase was obtained from Epicentre Technologies. [α-^{32}P]UTP and [α-^{32}P]dATP (3,000 Ci/mmol) were obtained from New England Nuclear. Unlabeled nucleoside triphosphates were purchased from Boehringer Mannheim.

DNA templates. Transcription reactions were programmed with DNA fragments isolated from variants of the plasmid pJX (28). The 450-bp transcription templates were prepared by isolating a 450-bp *Pvu*II fragment from this plasmid or its derivatives. Point mutations in the template (Fig. 1) were introduced by PCR mutagenesis as described previously (19).

Transcription in vitro. Transcription reactions were performed essentially as described previously (28). Briefly, 5 nM (each) DNA template was separately incubated with or without varying amounts of the 434 repressor for 10 min at 23°C in transcription buffer containing 100 mM KCl, 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, and 10 mM dithiothreitol. RNA polymerase was added to a final concentration of 50 nM, and incubation was continued for another 15 min at 37°C to allow the formation of open complexes. The transcription reaction was started by the addition of 0.25 mM ATP, GTP, or CTP; 0.04 mM UTP; 10 μCi of [α-^{32}P]UTP; and 0.1 mg of heparin per ml. After 10 min of further incubation, the reactions were stopped by addition of formamide dye mix (90% formamide) and fractionated on 6% denaturing gels. The amounts of RNA transcripts resulting from initiation at P_R and P_{RM} were quantified by PhosphorImager analysis.

DNase I footprinting. DNase I footprinting assays were performed essentially as described previously (28). Briefly, a 400-bp *Pvu*II-*Hin*dIII DNA fragment derived from the desired pJX derivative was 3′ end labeled using the Klenow fragment and [α-^{32}P]dATP. The DNA was mixed with increasing amounts of the 434 repressor in transcription buffer. After 10 min of incubation at 23°C, sufficient DNase I was added to give, on average, one cleavage per DNA molecule in 5 min of further incubation. The reaction products were precipitated with ethanol and *sec*-butanol, dissolved in a formamide dye, and resolved on 6% denaturing gels.

$KMnO_4$ footprinting. $KMnO_4$ footprinting was performed essentially as described previously (27). Briefly, the 400-bp *Pvu*II-*Hin*dIII DNA fragment was 3′ end labeled as described above. This fragment was incubated with or without varying concentrations of the 434 repressor at 23°C for 10 min, followed by the addition of RNA polymerase. After an additional 10-min incubation at 37°C, the DNA was exposed to 10 mM $KMnO_4$ for 1 min. The oxidation reaction was stopped by adding 1.3 M 2-mercaptoethanol, and the DNA was purified by two ethanol precipitations. The precipitated DNA fragments were dissolved in 100 μl of 1 M piperidine and incubated for 15 min at 90°C to induce cleavage at the modified bases. The DNA was then diluted in the same volume of ddH_2O, lyophilized twice, dissolved in formamide dye mix, and fractionated on a 6% denaturing polyacrylamide gel. The products were visualized by PhosphorImager analysis.

Gel mobility shift assay. The 400-bp *Pvu*II-*Hin*dIII DNA fragment isolated from pJX or its derivatives was 3′ end labeled as described above. The labeled DNA was mixed with increasing amounts of RNA polymerase in transcription buffer at 37°C and incubated for 15 min to allow the open complex formation. Subsequently, 0.1 mg of heparin per ml was added to remove nonspecifically bound RNA polymerases and/or closed promoter complexes before 5% glycerol was added prior to loading the sample onto a nondenaturing 3.5% polyacrylamide gel. The gels were run at 4°C with 0.5× Tris-borate-EDTA at 160 V for approximately 4 h. The gels were then dried, and the reaction products were visualized by PhosphorImager analysis.

RESULTS

Transcription from P_{RM} cannot be activated by the 434 repressor if RNA polymerase is added before the 434 repressor. The distance between the P_R and P_{RM} promoters in bacteriophage 434 is 17 bp less than in the related bacteriophage λ. Since, in both phages, the P_R promoter is substantially stronger than P_{RM} and an RNA polymerase-P_R complex interferes with open complex formation at P_{RM} in bacteriophage λ, we were interested in characterizing the predicted (1) promoter interference mechanism in bacteriophage 434. To begin this study, we compared the abilities of the 434 repressor to activate transcription from 434 P_{RM} when it is incubated with DNA prior to or after addition of RNA polymerase on a template that contains both the wild-type P_R and the P_{RM} promoters (Fig. 1). Consistent with previous reports (1, 2, 28), incubating the 434 repressor with the DNA template prior to the addition of RNA polymerase allows repressor-mediated activation of P_{RM} transcription and repression of P_R transcription (Fig. 2A). In contrast, activation of P_{RM} transcription by the 434 repressor is significantly reduced when the DNA template is first incubated with RNA polymerase at 37°C prior to adding the 434 repressor (Fig. 2B). Under the conditions of this experiment, RNA polymerase forms an open complex at P_R (1, 2). Apparently, the 434 repressor's ability to activate transcription from P_{RM} is inhibited by the formation of stable RNA polymerase open complexes at P_R.

Open complex formation on P_{RM} is inhibited if RNA polymerase is added before the repressor. The RNA polymerase-mediated "inhibition" of activated P_{RM} transcription shown in Fig. 2B could occur at any of the steps of the transcription pathway including closed complex and open complex formation at P_{RM} or transcription initiation and elongation processes. To distinguish between these possibilities, we observed open complex formation at P_{RM} by monitoring the $KMnO_4$ reactivity of the bases in the −10 region of this promoter. Since this method detects only unpaired thymines under the conditions used here, and since there are no accessible thymines in the labeled strand at P_R, this experiment monitors only open complex formation at P_{RM}. Incubating the 434 repressor with DNA template prior to adding RNA polymerase leads to a repressor-dependent activation of P_{RM} open complex formation. This finding is consistent with the repressor's ability to activate transcription from P_{RM} under these conditions (Fig. 2C). However, the ability of the 434 repressor to stimulate open complex formation at P_{RM} is almost completely eliminated if the template is incubated with RNA polymerase at 37°C prior to addition of the 434 repressor (Fig. 2D). Thus, RNA polymerase inhibition of repressor-mediated activation of P_{RM} transcription occurs prior to formation of an open complex. We note, however, that the inhibition of open complex formation is incomplete, in that some P_{RM} open com-

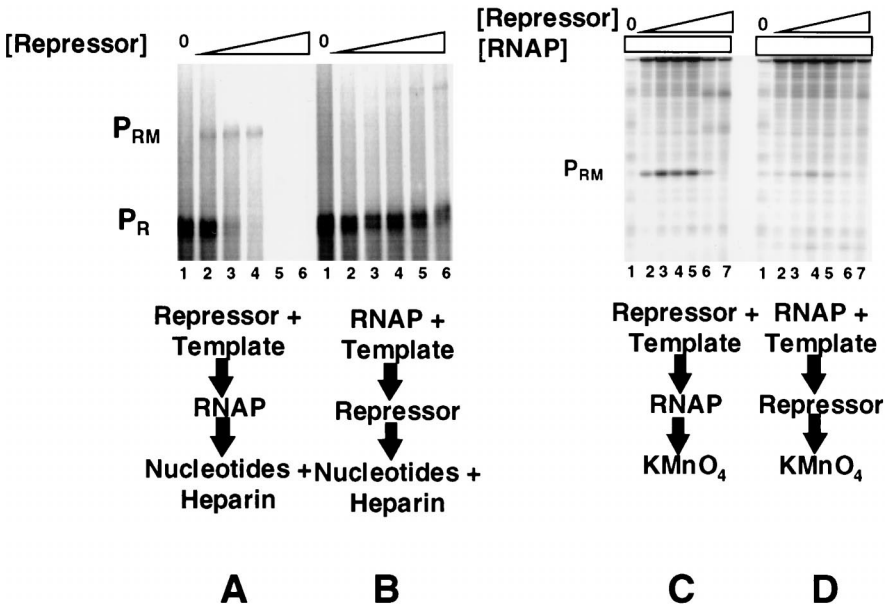

FIG. 2. Prior addition of RNA polymerase inhibits repressor-activated P_{RM} transcription (A and B) or open complex formation (C and D). For panels A and B, DNA fragments containing wild-type P_R and P_{RM} were transcribed in vitro in the absence of repressor (lanes 1) and at various increasing repressor concentrations. Repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein (lanes 2 to 6). Positions of transcripts resulting from initiation of transcription from P_{RM} and P_R are indicated. (A) The 434 repressor was incubated with DNA template at 23°C for 10 min, followed by addition of RNA polymerase. The reaction mixture was transferred to 37°C for 10 min before the transcription reaction was initiated by the addition of nucleotides and heparin. (B) RNA polymerase was incubated with DNA at 37°C for 10 min before addition of the 434 repressor. After an additional 10-min incubation at 37°C, the transcription reaction was initiated by the addition of nucleotides and heparin. (C and D) Shown are the open complexes formed at P_{RM} in the absence of repressor (lanes 1) and the presence of increasing concentrations of the 434 repressor as detected by $KMnO_4$ footprinting. Repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein. Footprinting conditions are given in Materials and Methods. Positions of P_R and P_{RM} open complexes are indicated. (C) Incubation conditions were as described for panel A. (D) Incubation conditions were as described for panel B. RNAP, RNA polymerase.

plexes are formed under these conditions. Since we do not observe any transcripts initiating at P_{RM} when the repressor is added to DNA after RNA polymerase (Fig. 2B), the observation of residual open complexes may indicate that the subsequent addition of the repressor also blocks P_{RM} promoter clearance.

The repressor binds to O_R2 in the presence of RNA polymerase bound at P_R. Since transcription initiation at P_{RM} requires a repressor-O_R2 complex (Fig. 2A), a simple explanation for the inhibitory effect of RNA polymerase on repressor activation of P_{RM} transcription would be that an RNA polymerase molecule at P_R prevents repressor binding. To test this idea, we examined the ability of the repressor to bind the sites in O_R in the absence or presence of RNA polymerase by DNase I footprinting. We first characterized the DNase I footprinting pattern of the repressor in the absence of RNA polymerase. Figure 3A shows that, in the absence of RNA polymerase, increasing 434 repressor concentrations result in progressive occupancy of the operator sites. The occupancy of these sites as a function of repressor concentration ($O_R1 \approx O_R2 > O_R3$) reflects their relative affinity for the repressor in intact O_R (25).

Next, we determined the DNase I footprinting pattern of the repressor-RNA polymerase-DNA ternary complex under the condition where RNA polymerase is added to DNA subsequent to the formation of the repressor-DNA complex. A difficulty with analyzing these footprinting results is that the repressor is able to both repress transcription of P_R and activate transcription of P_{RM} under these conditions (1, 2) (Fig. 2). Hence, the footprinting patterns reflect not only repressor binding but also the repressor's redirection of RNA polymerase binding from P_R to P_{RM}. Comparison of lanes 1 and 2 of Fig. 3B reveals that RNA polymerase fully or partially inhibits the DNAse I-mediated cleavage of numerous bases in the region of O_R that comprises the P_R promoter, from about +20 through −50 relative to the start site of P_R transcription (protected bases are labeled with solid circles in lane 2 of Fig. 3B). Addition of twofold-more RNA polymerase does not appreciably change the observed pattern of protection and enhancements (data not shown). Together with control $KMnO_4$ probe experiments (data not shown), these findings suggest that this pattern represents that of the RNA polymerase-P_R complex.

The observation that adding RNA polymerase to the repressor-DNA complex results in activation of P_{RM} transcription (Fig. 2A) suggests that these conditions should allow us to examine the DNase I footprint pattern of the repressor-RNA polymerase-P_{RM} ternary complex. Comparing lanes 1 and 2 with lanes 3 to 7 of Fig. 3B shows that adding increasing concentrations of the repressor followed by subsequent addition of RNA polymerase results in protection of several bases at the center of O_R2 and at either end of the O_R1 site (asterisk-marked bases in lane 3 in Fig. 3B) that are not protected by RNA polymerase alone. These additional protections result from occupancy of O_R1 and O_R2 by the repressor (see Fig. 3A for comparison). Inspection of the repressor-alone footprinting results in Fig. 3A shows that the repressor protects a region of DNA upstream of O_R1 that extends to position +20 of P_R from DNase I cleavage. This region is similarly protected in the presence of RNA polymerase bound at P_R (Fig. 3B, lane 2). However, when RNA polymerase is added to the repressor-DNA complex, this region is not protected but instead shows hyperreactive DNase I cleavage (positions marked with open arrowheads in Fig. 3B, lanes 3 to 7). These hyperreactive cleavages are not observed when RNA polymerase is added to DNA

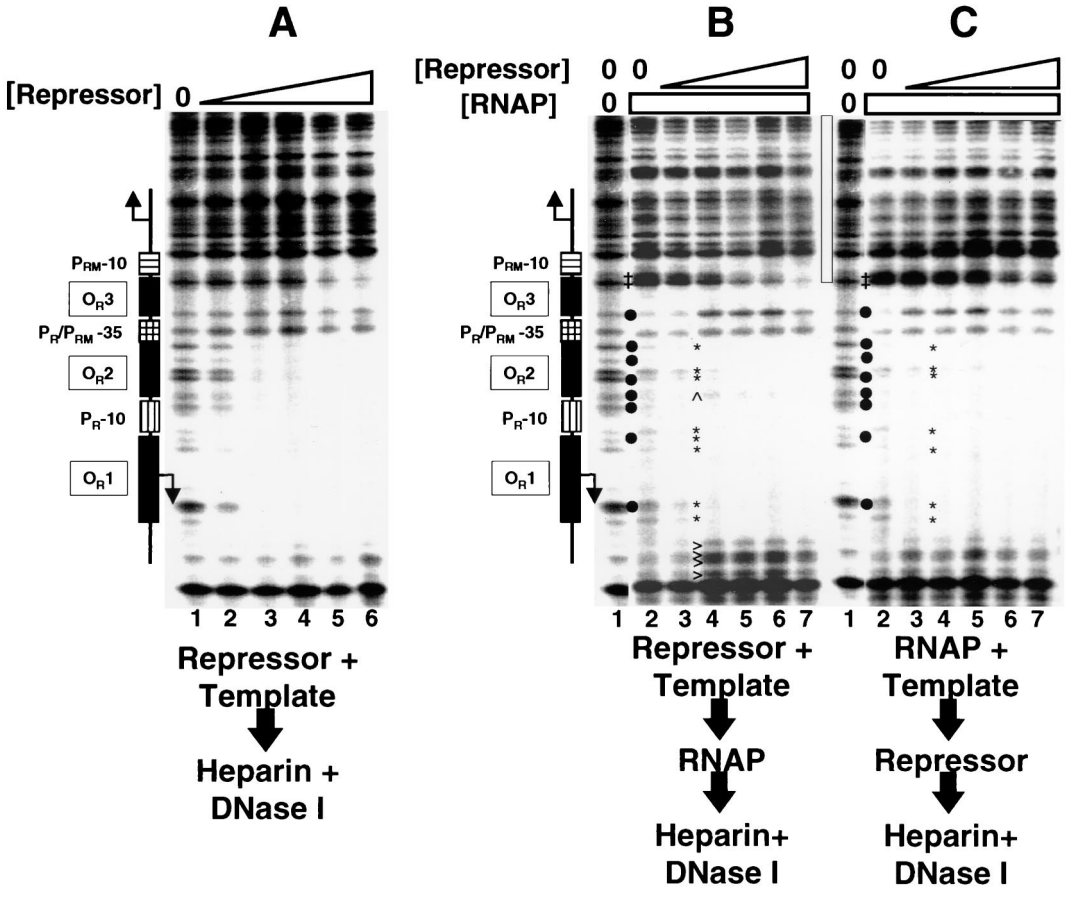

FIG. 3. The 434 repressor binds to O_R in the presence of RNA polymerase at P_R. DNA templates containing wild-type P_R and P_{RM} promoters were partially digested by DNase I in the presence of various amounts of the 434 repressor (A) or the 434 repressor and RNA polymerase (B and C). (A) Increasing concentrations of the repressor were incubated with DNA at 23°C for 10 min prior to addition of DNase I and heparin. Lane 1 shows the DNase I cleavage pattern of the DNA in the absence of added repressor. In lanes 2 to 6, repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein. (B and C) DNA and 50 nM RNA polymerase were incubated in the absence (lanes 1) or the presence of RNA polymerase (lanes 2) and the 434 repressor (lanes 3 to 7). The repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein. (B) In the lanes containing the repressor, the repressor was incubated with DNA template at 23°C for 10 min, followed by addition of RNA polymerase. The reaction mixture was transferred to 37°C for 10 min before the addition of DNase I and heparin. (C) RNA polymerase was incubated with DNA at 37°C for 10 min before addition of the 434 repressor (lanes 2 to 7). After an additional 10-min incubation at 37°C, cleavage was initiated by addition of DNase I and heparin. Positions of protection and enhancements resulting from protein binding to the sites indicated are denoted as described in the text. RNAP, RNA polymerase.

prior to adding the repressor (Fig. 3C, lanes 3 to 7). Adding RNA polymerase to the repressor-DNA complex also results in the appearance of a weak hypersensitive cleavage at position −15 of P_R (denoted by a caret), which is not seen when the repressor is added alone (compare lane 4 of Fig. 3A with that of 3B) or subsequent to RNA polymerase addition (Fig. 3C; see below). A stronger hypersensitive cleavage site is observed at position −30 of P_{RM}. In addition, several protections are observed in the −10 region of P_{RM} that are not well resolved on this gel (Fig. 3B, lanes 4 to 6, and data not shown). As supported by a similar analysis of template bearing a strong mutation in P_R (data not shown), we assert that this pattern of DNase I protections and enhancements represents that of the repressor-RNA polymerase-P_{RM} open complex (also Fig. 2C).

In light of the observation that the repressor is unable to activate transcription of P_{RM} if it is added to DNA after RNA polymerase (Fig. 2), a significant question is whether prior binding of RNA polymerase at P_R blocks repressor binding to its binding sites in O_R. Comparison of lanes 2 and 3 in Fig. 3C shows that adding the repressor to RNA polymerase bound at P_R results in the protection of bases at either end of O_R1, bases between O_R1 and O_R2 (denoted by asterisks in Fig. 3C), and bases at the center of O_R2 from DNase I digestion. These protections result from repressor binding and, moreover, occur at repressor concentrations that are very similar to those needed to occupy these sites in the absence of RNA polymerase. As a result of the overlap between the DNase I footprints of RNA polymerase-P_R complex and the complex of the repressor with O_R1 and O_R2, it is difficult to assess whether RNA polymerase remains bound to the template at the P_R promoter upon addition of the repressor. To answer this question, we compared the patterns of DNase I cleavage that are diagnostic for full repressor occupancy of O_R1 and O_R2 (Fig. 3A, lane 4) and of a repressor-RNA polymerase-P_{RM} open complex (Fig. 3B, lane 4) with the patterns present in Fig. 3C, lanes 3 to 6. The DNase I patterns of the samples in the latter lanes sample the structure of the complex under conditions where RNA polymerase is capable of transcribing P_R in the presence of the repressor (Fig. 2). The DNase I cleavage pattern of RNA polymerase alone (Fig. 3B and C, lanes 2) and complexes formed when RNA polymerase is added prior to the repressor (Fig. 3C, lanes 3 to 7) consistently display hypersensitive cleav-

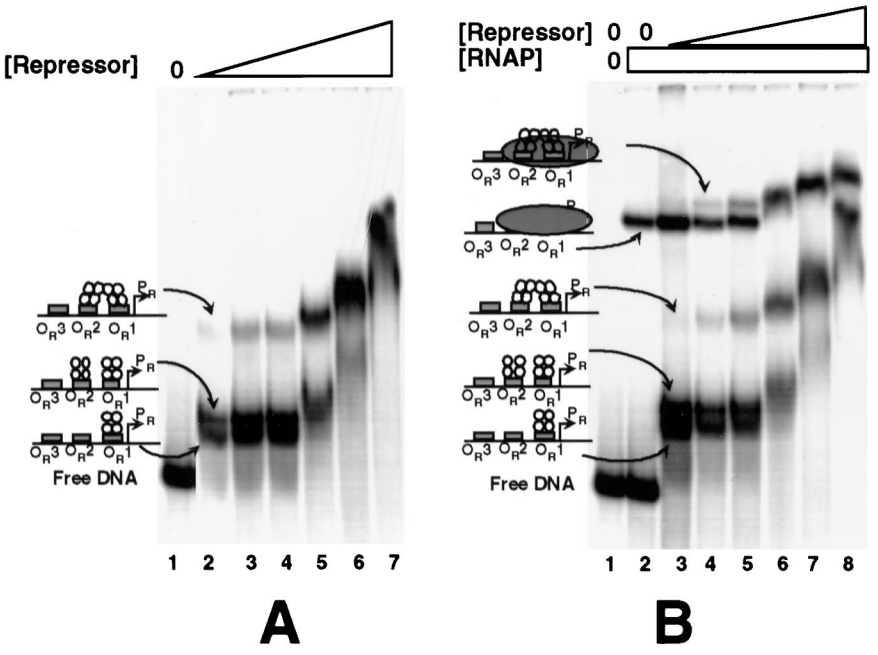

FIG. 4. The 434 repressor binds DNA in the presence of RNA polymerase at P_R. A DNA fragment containing wild-type O_R including the P_R and P_{RM} promoters was incubated with increasing concentrations of the 434 repressor in the absence (A) or the presence (B) of 50 nM RNA polymerase. Shown is a native gel of the resulting complexes. (A) O_R-containing DNA incubated in the absence (lane 1) or the presence (lanes 2 to 7) of increasing concentrations of the 434 repressor. The concentration of the repressor was increased in 2.5-fold steps starting at 100 nM. (B) O_R-containing DNA incubated in the absence (lane 1) or the presence of RNA polymerase (lane 2) or RNA polymerase and increasing concentrations of the 434 repressor (lanes 3 to 8). In lanes 3 to 8, RNA polymerase was added to the labeled DNA fragment, and this allowed the formation of an open complex at P_R by incubation for 10 min at 37°C. Subsequently, the repressor was added and the mixture was incubated at 37°C for an additional 10 min before addition of heparin and loading on the gel. RNAP, RNA polymerase.

ages at positions near −50 of P_R (lanes 2 of Fig. 3B and C, denoted by plus signs). These cleavages are absent in the repressor-only and the repressor-RNA polymerase-P_{RM} open complex lanes. This finding suggests that these cleavages are diagnostic for an RNA polymerase-P_R complex. Significantly, these hypersensitive cleavages are observed when the repressor is added and binds to O_R1 and O_R2 subsequent to the addition of RNA polymerase (Fig. 3C, lanes 3 to 6). This finding indicates that RNA polymerase remains bound at P_R when the repressor is added to DNA subsequent to RNA polymerase-P_R promoter complex formation. Moreover, the failure to detect protections in the −10 region of P_{RM} under these conditions suggests that RNA polymerase is unable to form a complex at P_{RM} under these conditions, even though the repressor is bound at O_R1 and O_R2.

In addition to the footprinting results shown in Fig. 3C, two additional lines of evidence also indicate that RNA polymerase is bound to P_R under the conditions of the experiments in Fig. 3C. First, under the same conditions, open complex formation at P_R is detected by $KMnO_4$ footprinting (see Fig. 5, below). Second, footprinting experiments performed with Cu(II) phenanthroline suggest that under these conditions RNA polymerase forms an open complex at P_R (data not shown). Third, RNA polymerase is capable of initiating transcription from P_R under these conditions (data not shown).

To further explore the potential for an effect of RNA polymerase-P_R complex formation on DNA binding by the 434 repressor, we monitored the formation of an RNA polymerase-repressor-DNA ternary complex by gel mobility shift assay. The results in Fig. 4A monitor the formation of 434 repressor-DNA complexes. At all repressor concentrations, we observe the formation of two sets of bands (Fig. 4A). DNase I footprinting studies and gel mobility shift experiments performed with repressor mutants that are unable to cooperatively bind O_R1 and O_R2 (data not shown) have allowed us to identify the nature of each of these species. The band with the lowest mobility represents a protein-DNA complex in which the repressor is bound at O_R1 and O_R2 and the repressors at the two sites are cooperatively interacting (Fig. 4A, lanes 2 to 7). The complex with the highest mobility represents the repressor dimer bound at O_R1 alone (Fig. 4A, lanes 2 to 4). The other, slightly lower mobility complex represents a DNA fragment on which the repressor is bound at O_R1 and O_R2 but the two repressors are not interacting, presumably because this interaction is disrupted during entry into the gel or during progression of the complex through the gel matrix (Fig. 4A, lanes 2 to 4). As a result of the very high protein concentrations in the samples in Fig. 4A, lanes 5 to 7, the mobilities of all these protein-DNA complexes decrease.

For the experiment in Fig. 4B, we first added RNA polymerase to the labeled DNA fragment. Closed complexes and nonspecifically bound RNA polymerase molecules were removed by heparin addition. This reaction results in the formation of a single band that corresponds to an RNA polymerase-P_R promoter complex (Fig. 4B, lane 2). Control experiments using a template bearing mutations in P_{RM} that prevent RNA polymerase from forming any complexes with P_{RM} formed an identical species, confirming that RNA polymerase is bound only at P_R under these conditions (data not shown; also Fig. 3B and C). Additionally, using $KMnO_4$ footprinting we confirmed that the only open complex formed under these conditions was at P_R (data not shown). Adding the 434 repressor to this mixture results in the formation of the same complexes identified in Fig. 4A. More importantly, the added repressor supershifts the band corresponding to the RNA polymerase-P_R promoter complex. Identical results were

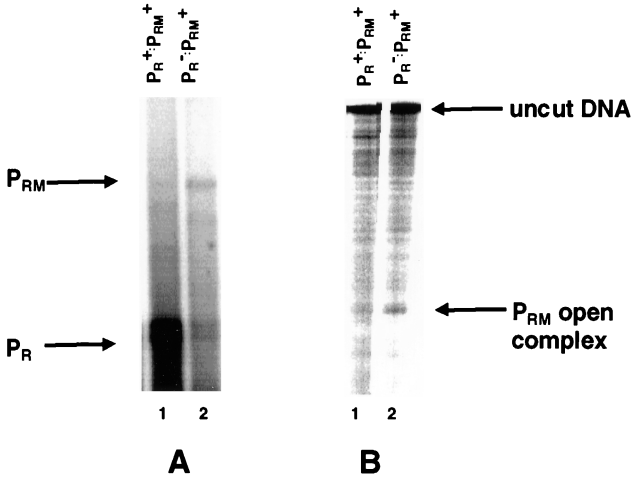

FIG. 5. Damaging P_R increases P_{RM} transcription initiation (A) and open complex formation at P_{RM} (B) in the absence of the 434 repressor. DNA containing a wild-type P_{RM} and a wild-type (lanes 1) or defective (lanes 2) P_R promoter was transcribed by RNA polymerase (A) or incubated with RNA polymerase and footprinted using $KMnO_4$ (B). The positions of the P_R and P_{RM} transcripts are indicated, as is the position of the P_{RM} open complex.

obtained with the template that bears mutations in P_{RM} that prevent RNA polymerase from forming a complex with P_{RM} (data not shown). These findings confirm that the 434 repressor is capable of binding to its operators even in the presence of RNA polymerase at P_R. Together, the results in Fig. 3 and 4 show that, in the presence of RNA polymerase bound at P_R, the 434 repressor is able to bind within O_R and that the repressor likely occupies both O_R1 and O_R2. This finding is somewhat surprising, considering that the P_R promoter sequence overlaps O_R1 and O_R2 (Fig. 1).

Only one open complex is allowed to form on the 434 O_R region. As discussed above, the −35 regions of the P_R and P_{RM} promoters substantially overlap (Fig. 1). This observation implies that the binding of RNA polymerase to the strong P_R promoter may directly interfere with RNA polymerase binding to the weaker P_{RM} promoter and that this direct interference may result in the repressor's inability to activate P_{RM} transcription initiation. To test this hypothesis, we compared the basal level of P_{RM} transcription and the amount of open complexes formed on a template bearing a defective P_R promoter with that found on a template bearing wild-type P_R. For this experiment, we mutated P_R by substituting a base pair at the −10 consensus region (see Fig. 1 for sequence). This mutation dramatically decreases the basal level of P_R transcription (Fig. 5A, compare lanes 1 and 2; see also Fig. 7 below). We find that, on the template bearing the mutant P_R promoter, approximately three times as many transcripts initiate at P_{RM} as on the template bearing the wild-type P_R promoter (Fig. 5A). Similarly, two- to threefold as many open complexes are formed at P_{RM} when P_R is defective as when it has wild-type activity (Fig. 5B). These data indicate that RNA polymerase bound at the P_R promoter interferes with open complex formation at P_{RM}.

Having established the existence of promoter interference between P_{RM} and P_R, we wished to establish the precise mechanism of interference. One possible mechanism is that, similar to the related λ phage, P_R interference with P_{RM} function may occur by inhibiting the rate of transition of the RNA polymerase-P_{RM} closed complex to an open complex (10, 26). If this hypothesis is correct, RNA polymerase should simultaneously form open complexes on both P_R and P_{RM} promoters. An alternative mechanism is that an open complex at P_R may simply prevent the formation of any RNA polymerase-P_{RM} complexes.

As the first step toward answering this question, we used gel mobility shift assays to determine how many open complexes can be formed on a single DNA template containing both 434 P_R and P_{RM}. We examined the ability of RNA polymerase to form open complexes on DNA templates bearing various arrays of wild-type and mutant P_R and P_{RM} promoters. Similar to Fig. 4, only one shifted band is observed when RNA polymerase is added to a template bearing both wild-type P_R and P_{RM} promoters (Fig. 6, lanes 2 to 4). This band is not observed with templates that bear a mutation that inhibits P_R open complex formation (data not shown; also Fig. 6, lanes 8 to 10). This finding establishes that the band seen in Fig. 6, lanes 2 to 4, represents a heparin-resistant RNA polymerase-P_R promoter complex.

We showed previously that a mutation that changes the sequence of the −35 region of 434 P_{RM} toward the consensus

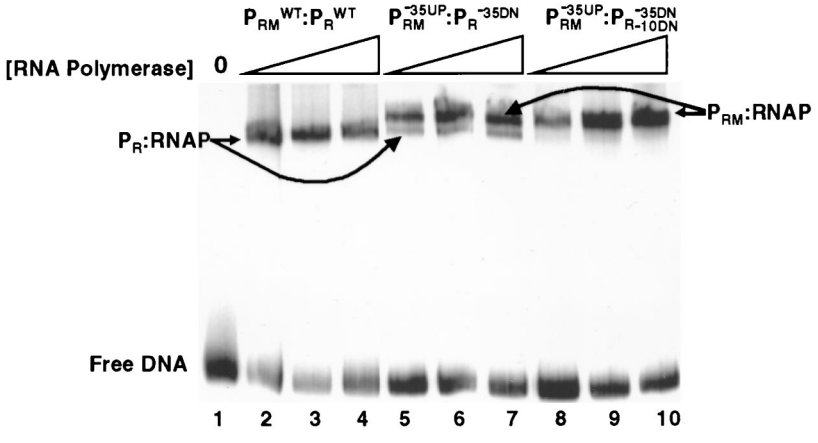

FIG. 6. Mutually exclusive binding of RNA polymerase to P_R and P_{RM}. Increasing concentrations of RNA polymerase were incubated with a DNA fragment containing wild-type P_R and P_{RM} (lanes 2 to 4), a fragment bearing a single mutation in the −35 region of P_R and P_{RM} that simultaneously decreases the strength of P_R and increases the strength of P_{RM} (lanes 5 to 7) (28), or a template bearing both the −35 mutation and a mutation in the −10 region of P_R (lanes 8 to 10; see Fig. 1 for sequences). The concentration of RNA polymerase was increased in threefold steps starting from 10 nM protein (lanes 2 and 8). RNA polymerase was added to the labeled DNA fragment incubated for 10 min at 37°C before addition of heparin and loading on the gel. RNAP, RNA polymerase; WT, wild type.

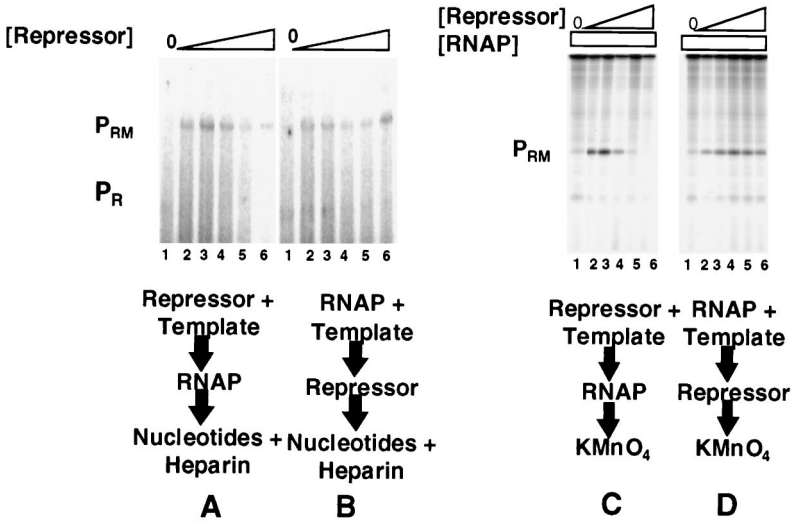

FIG. 7. Damaging P_R obviates RNA polymerase inhibition of repressor-activated P_{RM} transcription (A and B) or open complex formation (C and D). For panels A and B, DNA fragments containing wild-type P_{RM} and −10 mutant P_R (see Fig. 1 for sequence) were transcribed in vitro in the absence of repressor (lanes 1) and at various increasing repressor concentrations. Repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein (lanes 2 to 6). Positions of transcripts resulting from initiation of transcription from P_{RM} and P_R are indicated. (A) The 434 repressor was incubated with DNA template at 23°C for 10 min, followed by addition of RNA polymerase. The reaction mixture was transferred to 37°C for 10 min before the transcription reaction was initiated by the addition of nucleotides and heparin. (B) RNA polymerase was incubated with DNA at 37°C for 10 min before addition of the 434 repressor. After an additional 10-min incubation at 37°C, the transcription reaction was initiated by the addition of nucleotides and heparin. (C and D) Shown are the open complexes formed at P_{RM} in the absence of the repressor (lanes 1) and the presence of increasing concentrations of the 434 repressor as detected by $KMnO_4$ footprinting. Repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein. Footprinting conditions are given in Materials and Methods. Positions of P_R and P_{RM} open complex are indicated. (C) Incubation conditions were as described for panel A. (D) Incubation conditions were as described for panel B. RNAP, RNA polymerase.

sequence (Fig. 1) increases initiation from P_{RM} and decreases transcription initiation from P_R (28). When RNA polymerase is incubated with this template, the results in Fig. 6, lanes 5 to 7, show that two shifted bands are observed. Since $KMnO_4$ footprinting data indicate that open complexes are formed at P_R and P_{RM} (data not shown), we suggest that each band represents an open complex formed at P_{RM} or P_R, respectively, on separate DNA molecules.

These findings do not definitively prove that each band represents an individual open complex at P_{RM} and P_R, which are formed on two different DNA molecules. It is formally possible that the higher-mobility band represents an RNA polymerase-DNA complex at a single promoter, while the complex having lower mobility represents an RNA polymerase complex at both P_{RM} and P_R on the same DNA fragment. Alternatively, it is possible that the two bands observed in Fig. 6, lanes 5 to 7, represent two forms of a complex formed at a single promoter. To begin to distinguish these possibilities, we examined the ability of RNA polymerase to form heparin-resistant complexes on a template bearing two mutations, the combined consequences of which increase the strength of P_{RM} and decrease the strength of P_R. The first mutation is located within the −35 region of P_{RM} that simultaneously increases the match of its −35 region of 434 P_{RM} with the consensus sequence but decreases the match of the −35 region of P_R to the consensus sequence. The second mutation is in the −10 region of P_R (see Fig. 1 for sequences). This change, combined with the −35 alteration, renders the P_R promoter incapable of forming any complexes with RNA polymerase. $KMnO_4$ probe experiments show that, on this template, RNA polymerase forms only open complexes at P_{RM} (data not shown). The results in Fig. 6, lanes 8 to 10, show that only one shifted band is observed when RNA polymerase is incubated with this template. The mobility of this complex is identical to that of the lower-mobility species seen in Fig. 6, lanes 5 to 7. This finding indicates that the lower-mobility complex does not contain a DNA molecule bearing an RNA polymerase at both P_{RM} and P_R. Since only a single species is formed on this template, this finding also suggests that the two species that are observed in Fig. 6, lanes 5 to 7, do not represent two forms of the same RNA polymerase-promoter complex. Moreover, since only the lower-mobility complex forms on this template, and since this complex is observed only when RNA polymerase forms an open complex at P_{RM}, we suggest that this complex is the RNA polymerase-P_{RM} open complex. Hence, the lower- and higher-mobility complexes formed under the conditions of the experiment in Fig. 6, lanes 5 to 7, represent open complexes formed at P_{RM} and P_R, respectively, on separate DNA molecules. Most importantly, these observations indicate that formation of open complexes at P_{RM} and P_R promoters is mutually exclusive. Based on the analysis of the data in Fig. 6, if open complexes were allowed to simultaneously form on both P_R and P_{RM} promoters, a third, higher-molecular-weight species should be observed.

Role of P_R sequence in inhibiting activation of P_{RM} transcription. The above results demonstrate that prior addition of RNA polymerase decreases the repressor's ability to stimulate open complex formation at P_{RM} by prohibiting the access of RNA polymerase to the P_{RM} promoter sequence. We wished to determine what kind of RNA polymerase-P_R complex is capable of inhibiting repressor-mediated activation of P_{RM} transcription. As a first step in this investigation, we examined whether the relative strength of the P_R promoter plays a role in inhibiting the activation of P_{RM} open complex formation by the 434 repressor. This experiment employs the −10 mutant P_R promoter used in the experiments presented in Fig. 5 and 6 (see Fig. 1 for sequence). The −10 sequence change dramatically decreases the transcriptional activity of the P_R promoter (Fig. 7A, lane 1). Similar to the results obtained using a template bearing the wild-type promoters (Fig. 2A), the 434 re-

pressor is able to activate transcription from P_{RM} when it is added to the template prior to RNA polymerase (Fig. 7A). However, in contrast to the results obtained with the wild-type promoters, the 434 repressor is also able to activate P_{RM} transcription even if RNA polymerase is incubated with DNA template prior to adding the repressor (Fig. 7B; also compare these results to those shown in Fig. 2B). The failure of the mutant P_R to inhibit initiation of transcription from P_{RM} under these conditions may result from a decrease in the lifetime of the mutant P_R-RNA polymerase open complex or an inability of the mutant promoter-RNA polymerase complex to interfere with repressor function (see Discussion). Nonetheless, these findings suggest that the RNA polymerase-mediated inhibition of P_{RM} transcription requires a strong P_R promoter.

$KMnO_4$ footprinting experiments were performed to confirm that the loss of the inhibition, resulting from weakening P_R by mutation, affects P_{RM} open complex formation. The results in Fig. 7 show that, on a template bearing a mutant P_R promoter, the maximal amount of P_{RM} open complex is formed regardless of whether RNA polymerase is added after (Fig. 7C) or before (Fig. 7D) the 434 repressor. These results differ from those obtained on templates bearing the wild-type P_R promoter (Fig. 2C and D) and confirm that decreasing the strength of P_R relieves the RNA polymerase-mediated inhibition of the repressor-stimulated P_{RM} open complex formation. Together with the results in Fig. 4 and 5, these results suggest that RNA polymerase-mediated inhibition of P_{RM} transcription occurs at the level of RNA polymerase binding to P_{RM}.

An open complex formed at P_R is required for the inhibition of P_{RM} activity. The foregoing experiments demonstrate that changing the strength of P_R can modulate the efficiency of repressor-mediated P_{RM} activation. However, these experiments do not provide information regarding the molecular basis for this modulation. We took advantage of the fact that open complex formation is temperature dependent to examine whether an open complex engaged at the P_R promoter is required to prevent repressor-mediated activation of P_{RM} transcription. We have established that only closed, not open, complex formation can proceed at low (0 to 5°C) temperatures (data not shown) and that efficient open complex formation at P_R requires temperatures in excess of 12°C. Thus, if formation of an open complex at P_R is required for the inhibition of P_{RM} activation, incubation of RNA polymerase at 0°C before adding the repressor should not prevent activation of P_{RM} by the repressor. The results in Fig. 8 show that, in contrast to the results obtained at higher temperatures, incubating RNA polymerase and the DNA fragment at 0°C prior to adding the repressor does not inhibit transcription activation of P_{RM} by the 434 repressor. This finding indicates that formation of open complex at the P_R promoter is required for the inhibition of 434 repressor-mediated activation of P_{RM}.

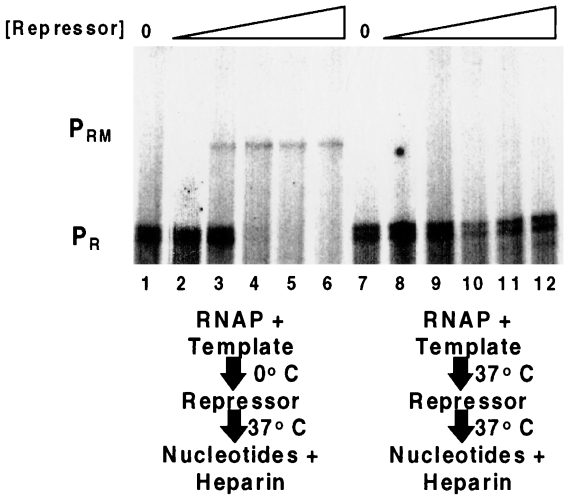

FIG. 8. An open complex at P_R is required for the inhibition of repressor-mediated activation of P_{RM}. A DNA fragment containing wild-type P_R and the P_{RM} promoter was transcribed in vitro in the presence of the repressor. Transcription conditions are given in Materials and Methods. Repressor concentrations were increased in 2.5-fold steps starting at 250 nM protein. Positions of transcripts resulting from initiation of transcription from P_{RM} and P_R are indicated. RNAP, RNA polymerase.

DISCUSSION

Our data clearly demonstrate that open complex formation at the P_R promoter prevents open complex formation on P_{RM}. This finding indicates that, in 434 O_R, 434 repressor-mediated activation of P_{RM} transcription occurs through two mechanisms. First, the 434 repressor stimulates open complex formation at P_{RM} by directly contacting RNA polymerase (2, 3, 28). Second, in agreement with the suggestions of others (1), the results shown in this paper indicate that the 434 repressor also activates P_{RM} transcription by releasing an "inhibitory" effect of RNA polymerase bound at the strong promoter P_R. The homologous λ repressor employs identical strategies in stimulating transcription from λ P_{RM} (6, 10, 11, 12, 14, 16, 18). The congruence of mechanisms used in stimulating P_{RM} in these two phages supports the assertion that these strategies may be used generally in all homologous phages (10).

Although the activities of the P_{RM} promoters in bacteriophages λ and 434 are regulated by interference between P_R and P_{RM}, the specific mechanisms by which RNA polymerase at P_R inhibits P_{RM} transcription initiation appear to differ between the two phages. In the case of the λ repressor, an open complex at P_R does not appear to affect binding of RNA polymerase to P_{RM} (10). Instead, the RNA polymerase-P_R complex inhibits the rate of isomerization of a closed complex at P_{RM} to an open complex (6, 11, 26). Hence in λ O_R, open complex formation at P_R and that at P_{RM} are not mutually exclusive. We are unable to detect a ternary complex in which both 434 P_R and P_{RM} are occupied (Fig. 6), indicating that in 434 O_R open complex formation at P_R prevents formation of a similar complex at P_{RM}.

A comparison of sequences of the O_R regions of the two phages suggests a reason for the difference in their promoter exclusion mechanisms. In λ O_R, the −35 regions of P_R and P_{RM} are located to the right and left of O_R2, respectively, and are separated from each other by 12 bp. In 434 O_R, the −35 regions of P_R and P_{RM} virtually overlap on the left side of O_R2. Hence, simultaneous occupancy of each promoter by RNA polymerase is forbidden by steric occlusion. To better understand the spatial relationships among the proteins that control the activities of P_R and P_{RM}, an unwrapped cylindrical projection of the O_R region that identifies the positions of the phosphates actually or presumed to be contacted by the repressor and RNA polymerase is presented in Fig. 9 (2, 22). This DNA projection indicates that most of the phosphates contacted by the repressor bound at O_R1 and O_R2 are not contacted by RNA polymerase bound at the P_R promoter. In addition, this model also indicates that RNA polymerase bound at the P_R promoter and the repressor bound at O_R1 and O_R2 are essentially located on different faces of the DNA. These inferences are consistent with the results showing that the repressor at these sites and RNA polymerase at P_R can coexist simulta-

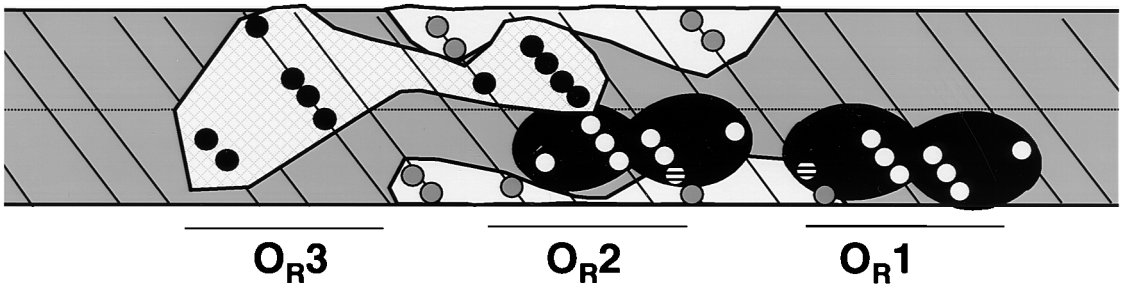

FIG. 9. Disposition of RNA polymerase and the 434 repressor bound at the 434 O_R region. Ethylation interference data are mapped onto an unwrapped cylindrical projection of the surface of a DNA double helix, assuming 10.5 bp per turn. Ethylation interference patterns for RNA polymerase bound at the P_R promoter are deduced from data obtained for the T7A3 and PlacUV5 promoters (22). Ethylation interference patterns for RNA polymerase at the 434 P_{RM} promoter and the 434 repressor at O_R1 and O_R2 are based on the data in reference 2. The black area represents the 434 repressor. The white area represents RNA polymerase at P_R, and the hatched area denotes the position of RNA polymerase at P_{RM}. Phosphate contacts for each protein are denoted by white circles (repressor at O_R1 and O_R2), black circles (RNA polymerase at P_{RM}), and gray circles (RNA polymerase at P_R). The hatched circles denote phosphates that could be contacted by RNA polymerase and/or the 434 repressor.

neously on DNA. Examination of Fig. 9 also shows that RNA polymerase binding blocks the access of the repressor to phosphate at 3′ to the base at position 10 of O_R2. This finding suggests that prior binding of RNA polymerase at P_R may alter the structure of the repressor-O_R2 complex. This putative structural alteration could also contribute to RNA polymerase-mediated inhibition of repressor-stimulated P_{RM} transcription. Similarly, this model predicts that RNA polymerase may be able to form a nontranscriptionally active complex with P_R in the presence of the repressor bound at O_R2. Data obtained by our laboratory support both of these ideas (J. Xu and G. B. Koudelka, unpublished data).

Since inactivating P_R allows RNA polymerase to initiate transcription at the weak P_{RM} promoter (Fig. 5), we are interested in assessing the relative contribution of the indirect effect of relieving promoter competition to the overall efficiency of the 434 repressor activation of P_{RM} transcription. The work of Gussin and coworkers (26) with λ phage suggests that interference between λ P_{RM} and λ P_R does not limit the rate of open complex formation at λ P_{RM} in the cell. Apparently, rapid transcription initiation clears both the λ P_R and λ P_{RM} promoters rapidly enough that neither is occupied for a significant fraction of the time, thereby minimizing the effects of promoter interference in vivo. Although we do not have direct evidence, correlation between the in vivo and in vitro studies of 434 promoter utilization and the data presented in this paper suggest that promoter interference may have a role in vivo in the 434 bacteriophage. Overall, adding the repressor increases the amount of open complexes and transcripts from P_{RM} by 10-fold (2, 28). The effect of mutating P_R increases the amount of runoff transcripts by about threefold and the amount of open complexes by a similar amount (Fig. 5). This analysis indicates that repressor-mediated relief of promoter competition contributes nearly as much to the 434 repressor's activation of P_{RM} transcription as does direct stimulation of RNA polymerase. Consistent with the relative importance of the indirect stimulation mechanism, positive control mutants that are presumably defective in directly contacting RNA polymerase stimulate P_{RM} transcription at least half as well as does the wild-type repressor in vivo (2). This finding also indicates that relief of promoter interference may have a significant role in regulating P_{RM} transcription in the bacteriophage. Further support for this view comes from the finding that eliminating RNA polymerase binding at P_R by 434 Cro binding to O_R1 and/or O_R2 also stimulates transcription from P_{RM} (1). Similarly, deletion of P_R increased transcription from P_{RM} threefold in vitro (2).

Kinetic assays indicate that the direct stimulation of λ P_{RM} by the λ repressor occurs by increasing the rate of isomerization of RNA polymerase from a closed to an open complex at λ P_{RM} (7, 8). Relief of promoter interference by the λ repressor also leads to an increase in the rate of isomerization (6, 11, 26). Although the precise kinetic mechanism by which the 434 repressor stimulates transcription from 434 P_{RM} has not yet been determined, several lines of evidence indicate that the 434 repressor enhances the formation of closed complexes by recruiting RNA polymerase to the P_{RM} promoter (28). Similarly, the 434 repressor-mediated relief of promoter competition would also be expected to result in an increase in the number of closed complexes at P_{RM}. The variance in overall mechanism of activation in these two phages is likely related to promoter sequence-dependent differences in the identity of the rate-limiting steps between the two P_{RM} promoters and not to a difference in the stimulatory properties of the two repressors (21). This idea is supported by the observation that the λ repressor can stimulate closed complex formation by a mutant RNA polymerase (15). Moreover, the λ repressor is also able to activate transcription simply by providing an arbitrary protein-protein contact with RNA polymerase (5).

We have shown that open complex formation at the P_{RM} promoter is inhibited by open complex formation on P_R. With this observation in mind, one question still remains. Given that an open complex on P_R appears to be required to inhibit transcription from P_{RM}, how can 15 to 20% of DNA that forms an open RNA polymerase-P_R complex cause the 80% decrease in P_{RM} transcription (Fig. 5)? One possible answer is that these complexes do not represent all of the heparin-resistant DNA-RNA polymerase complexes. It is possible that these intermediate complexes account for a reasonably large portion of the population and enforce an inhibitory effect on P_{RM} transcription.

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