Sequence-Dependent Upstream DNA–RNA Polymerase Interactions in the Open Complex with $\lambda P_R$ and $\lambda P_{RM}$ Promoters and Implications for the Mechanism of Promoter Interference

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Upstream interactions of Escherichia coli RNA polymerase (RNAP) in an open promoter complex (Rpo) formed at the $P_R$ and $P_{RM}$ promoters of bacteriophage $\lambda$ have been studied by atomic force microscopy. We demonstrate that the previously described 30-nm DNA compaction observed upon RPo formation at $P_R$ [Rivetti, C., Guthold, M. & Bustamante, C. (1999). Wrapping of DNA around the E. coli RNA polymerase open promoter complex. EMBO J., 18, 4464–4475.] is a consequence of the specific interaction of the RNAP with two AT-rich sequence determinants positioned from $−36$ to $−59$ and from $−80$ to $−100$. Likewise, RPo formed at $P_{RM}$ showed a specific contact between RNAP and the upstream DNA sequence. We further demonstrate that this interaction, which results in DNA wrapping against the polymerase surface, is mediated by the C-terminal domains of $\alpha$-subunits (carboxy-terminal domain). Substitution of these AT-rich sequences with heterologous DNA reduces DNA wrapping but has only a small effect on the activity of the $P_R$ promoter. We find, however, that the frequency of DNA templates with both $P_R$ and $P_{RM}$ occupied by an RNAP significantly increases upon loss of DNA wrapping. These results suggest that $\alpha$ carboxy-terminal domain interactions with upstream DNA can also play a role in regulating the expression of closely spaced promoters. Finally, a model for a possible mechanism of promoter interference between $P_R$ and $P_{RM}$ is proposed.

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Introduction

The bacteriophage $\lambda$ promoters $P_R$ and $P_{RM}$ control the expression of repressor proteins cro and cl, which drive the establishment of the lytic and lysogenic states.$^1$ $P_R$ and $P_{RM}$ are divergently transcribed from start sites separated by a 82-bp sequence harboring the $−10$ and $−35$ hexamers of both promoters and the operator sites $O_R1$, $O_R2$, and $O_R3$. Differential binding of cl to the three operator sites represses $P_R$ and regulates $P_{RM}$ either positively or negatively. Conversely, differential binding of cro to the operator sites represses $P_{RM}$ and, at higher concentrations, turns down its own synthesis by repressing $P_R$ as well.$^{3,4}$ Open promoter complex (Rpo) formation at $P_R$ is much faster than open complex formation at $P_{RM}$; thus, the binding of an RNA polymerase (RNAP) to $P_{RM}$ only takes place in the context of an RNAP bound to $P_R$.$^{5,6}$ It has been shown that $P_{RM}$ activity is increased by mutations designed to inactivate $P_R$ and, conversely, an
Fig. 1. (a) Sequences of upstream regions of the λPR and λPRM variants used in this work. The transcription start site +1, the −10 and −35 hexamers are singly underlined for PR and doubly underlined for PRM. ATR regions are shown in purple boldface type. Heterologous DNA is shown in lowercase blue type. Point mutations are shown in boldface type. (b) AFM image of RPos formed with wt RNAP and the wt PR–wt PRM DNA template. (c) AFM image of RPos formed with ΔαCTD\textsuperscript{II} RNAP and the wt PR–wt PRM DNA template. The images scan size is 2 μm.
RNAP bound to P_{RM} interferes with the utilization of a weakened P_R promoter.\textsuperscript{14} Interestingly, it has been shown that P_R and P_{RM} promoters can be occupied simultaneously by an RNAP, and this has led to the conclusion that formation of an open complex at P_R does not prevent binding of an RNAP to P_{RM}, but rather impairs isomerization from a closed complex to an open complex.\textsuperscript{7-10} Contrary to expectations, a 10-bp deletion between the −35 elements of the two promoters, which should reduce the space available for two polymerases without affecting promoter sequences, resulted in relief of interference.\textsuperscript{12,13} Studies that aimed to elucidate the structure of the AP_R open complex have shown that, at variance with other promoters, DNA is tightly wrapped around the RNAP, extending the RNAP–DNA interaction further upstream of the P_R −35 element.\textsuperscript{14–16} This extended contact is generally mediated by α-subunit carboxy-terminal domains (CTDs) that interact with A + T-rich (ATR) DNA sequences located upstream of the promoter.\textsuperscript{15–19} This extended upstream DNA interaction observed at P_R prompted us to search for possible upstream sequence motifs that might specifically interact with the RNAP and to further investigate their effect on P_R activity and P_{RM} accessibility in the context of a P_R-bound RNAP. To this end, we have employed atomic force microscopy (AFM) to image RPos formed at P_R at P_{RM}, and at variants of these promoters in which the upstream regions were selectively substituted with heterologous DNA. Comparison of the DNA contour length of RPo with that of free DNA molecules revealed previously unrecognized upstream DNA–RNAP interactions at the P_R promoter that might explain not only the strong DNA compaction typical of RPo formed at this promoter but also the previously described P_R–P_{RM} promoter interference.

### Results

To assess the involvement of ATR regions positioned upstream of P_R in the DNA compaction of Rpo, we have constructed P_R promoter variants in which the upstream DNA was selectively substituted with heterologous DNA, and we have measured the DNA compaction of the corresponding RPo. Figure 1a reports the DNA sequence of the promoter variants used in this study, and Fig. 1b shows representative AFM images of RPo. RPos were formed by incubating DNA and RNAP in a 1:1 molar ratio in transcription buffer [20 mM Tris–HCl (pH 7.9), 50 mM KCl, 5 mM MgCl₂, and 1 mM DTT] at 37 °C for at least 20 min. RPo activity was verified by runoff transcription assays conducted under similar conditions, but with the addition of heparin to prevent multiple rounds of transcription (Fig. 2). Heparin could not be used in the AFM experiments because it prevents adhesion of the DNA to mica support; thus, the RPos, seen microscopically, were considered such because they were formed under conditions favoring open complex formation and were located at the promoter site.

### DNA compaction at P_R and P_{RM} depends on upstream sequence determinants

The wild-type (wt) DNA template harbors both P_R and P_{RM} promoters; however, RNAP primarily binds to P_R, as demonstrated by the outcome of single-round transcription experiments in which only a transcript from P_R is observed (Fig. 2, lane 1) and by previous work.\textsuperscript{5–7,11} Therefore, complexes assembled with this DNA template were considered as RPo formed at P_R. DNA contour length analysis of these complexes shows an average DNA compaction of 30 nm (Fig. 3a and Table 1), in full agreement with previously published data.\textsuperscript{14,15} A 30-nm DNA compaction was also observed with P_R (−100 to +34) (Fig. 3b and Table 1), in which the DNA sequence upstream of −100 was substituted with vector DNA. Thus, the DNA sequence upstream of position −100 is not involved in the DNA compaction observed at P_R (i.e., within the Rpo, this sequence is not contacted by RNAP).

Conversely, RPo formed with P_R (−79 to +34), in which the DNA sequence upstream of −79 was substituted with vector DNA, shows 10-nm DNA compaction of 20 nm (Fig. 3c and Table 1). This result indicates that in the P_R region from −80 to −100, there must be a sequence determinant that is specifically contacted by an RNAP bound at P_R.

![Fig. 2. Single-round in vitro transcription of the DNA templates shown in Fig. 1. Lane 1, wt P_{K}–wt P_{RM}; lane 2, P_R (−100 to +34); lane 3, P_R (−79 to +34); lane 4, P_R (−99 to +34); lane 5, P_R (−35 to +34); lane 6, P_R–wt P_{RM}; lane 7, P_{RM} (−35 to +541). All transcription reactions were carried out in the presence of heparin. The two bands of the P_R transcript are probably due to inhomogeneous runoff termination. The P_{RM} transcripts in lanes 6 and 7 have different lengths because of the different lengths of the downstream DNA. The relative band intensity is reported below each lane: lanes 2–5 with respect to lane 1, and lane 7 with respect to lane 6.](image-url)
Because a DNA compaction of 20 nm is still significantly high compared to those of other promoters,\textsuperscript{15} we hypothesized that other sequence determinants that are capable of interacting with the RNAP must be present in the upstream sequence of P\textsubscript{R}. RPo formed with P\textsubscript{R} (−59 to +34), in which the DNA sequence upstream of −59 was substituted with vector DNA, again shows a DNA compaction of 20 nm (Fig. 3d and Table 1). Thus, the DNA region from −60 to −79 does not contain sequence determinants involved in DNA compaction. However, substitution of the DNA sequence upstream of −35, as in the case of P\textsubscript{R} (−35 to +34), resulted in a DNA compaction of only 4 nm. This reduced extent of DNA compaction indicates the absence of stable interaction between the RNAP and upstream DNA, and suggests that a second sequence determinant, specifically recognized by the RNAP, is present in the P\textsubscript{R} region from −36 to −59.

A similar analysis was conducted to investigate the RNAP interaction in RPo formed at P\textsubscript{RM}. To avoid competition, the stronger P\textsubscript{R} promoter was inactivated by mutating the −10 hexamer in a manner known to reduce P\textsubscript{R} activity to 2–5% of wt (GATAAT changed to GGTGAC; Fig. 1a).\textsuperscript{20} Single-round \textit{in vitro} transcription indeed shows that transcription of the P\textsubscript{R}−wt P\textsubscript{RM} construct produces a 154-nt transcript from P\textsubscript{RM} and no detectable transcription from P\textsubscript{R} (Fig. 2, lane 6). Thus, RPos assembled with this template were assumed to be formed at P\textsubscript{RM}. DNA contour length measurements of these RPos revealed a DNA compaction of 18 nm (Fig. 4a and Table 1), which is an indication of an extended interaction of the RNAP with upstream DNA. The DNA compaction observed at P\textsubscript{RM} is significantly less than that observed at P\textsubscript{R} and is comparable to the DNA compaction observed at P\textsubscript{R} (−59 to +34) and \textit{LacUV5} (UPfull) in which an UP element has been placed in the −38/−59 region of \textit{LacUV5}.\textsuperscript{15} Thus, based on DNA compaction similarity, we hypothesized that the RNAP–DNA interaction at P\textsubscript{RM} might involve DNA sequences in the P\textsubscript{RM} region from −36 to −60.

In order to determine whether sequence determinants in the upstream region of P\textsubscript{RM} contribute to the observed compaction, we constructed P\textsubscript{RM} (−35 to +541), a λP\textsubscript{RM} derivative in which the sequence upstream of −35 has been replaced with heterologous DNA (Fig. 1a; the P\textsubscript{R} promoter −10 and −35 hexamer regions are deleted in this

**Fig. 3.** DNA contour length distributions of bare DNA and RPo formed with wt RNAP and P\textsubscript{R} derivatives as follows: (a) wt P\textsubscript{R}−wt P\textsubscript{RM}; (b) P\textsubscript{R} (−100 to +34); (c) P\textsubscript{R} (−79 to +34); (d) P\textsubscript{R} (−59 to +34); (e) P\textsubscript{R} (−35 to +34). In all panels, white dashed bars represent bare DNA contour length frequencies, and dark-gray bars represent RPo contour length frequencies. Solid lines represent Gaussian fitting of the distributions. Mean and standard error values derived from the fitting are reported in Table 1. DNA compaction, defined as the difference between the means of DNA and RPo distributions, is reported on each graph.
construct). A contour length analysis of these complexes is shown in Fig. 4b and Table 1. RPo at \( P_{RM} \) (−35 to +541) displays a DNA compaction of 8 nm, thus confirming the presence of a sequence determinant in the upstream region of \( P_{RM} \) that is capable of making specific interactions with the RNAP.

**Effects of upstream DNA determinants on \( P_{R} \) and \( P_{RM} \) promoter function**

Two types of experiments, in vitro transcription and RNAP promoter association rate measurements, were carried out to determine whether the substitutions for upstream promoter DNA described above (Fig. 1a) affect the functional interaction of RNAP with the \( P_{R} \) or \( P_{RM} \) promoters. The substitution for sequences upstream of −100 in \( P_{R} \) did not alter its in vitro transcription activity relative to that of the wt template (Fig. 2, lanes 1 and 2). However, substitutions for upstream regions closer to the \( P_{R} \) promoter resulted in small increases in \( P_{R} \) expression (~1.7- to 2.7-fold greater than wt for the −79, −59, and −35 substitutions; Fig. 2, lanes 3–5). These results suggest the possibility of a small inhibitory effect of the wt upstream regions on expression from \( P_{R} \). However, the observed increases in transcription did not correlate with any effect on the overall rates of RNAP association with these \( P_{R} \) promoters (\( k_{i} \); see below), suggesting that the mechanism of the increases may involve steps after RNAP promoter complex formation. Inhibitory effects of ATR upstream sequences on promoter clearance have previously been noted for some promoters.21 We note that the small increases in transcription did not correlate with loss of the \( P_{RM} \) −10 and −35 sequences, since the fragment retaining these sequences (\( P_{R} \) −79 to +34) also showed a small increase in \( P_{R} \) transcription.

Transcription from the \( P_{RM} \) promoter was not detected for any of the promoter region fragments containing a \( P_{R} \) promoter (including, as expected, those for fragments −79, −59, and −35, in which all or part of \( P_{RM} \) was deleted; Fig. 2, lanes 1–5). However, transcription from \( P_{RM} \) was observed with a fragment containing a substitution in the −10 region of \( P_{R} \) (\( P_{R} \)–wt \( P_{RM} \) in Fig. 1a; lane 6 in Fig. 2). The substitution for sequences upstream of the −35 region of \( P_{RM} \) [\( P_{RM} \) (−35 to +541); Fig. 1a] removed the \( P_{R} \) promoter and resulted in a small increase in \( P_{RM} \) transcription (~2-fold; Fig. 2, compare lanes 6 and 7). (The size of the RNA transcripts in lanes 6 and 7 is different because the two promoter fragments have different lengths of DNA downstream of the \( P_{RM} \) promoter.)
DNA wrapping at $P_R$ does not affect the overall association and isomerization rate constants

By comparing the rates of association of RNAP with $\lambda P_R$ fragments containing different lengths of upstream DNA (a “full-length” fragment with upstream DNA up to −110, and an upstream truncated fragment with upstream DNA only up to −47), it was shown that the presence of upstream DNA greatly increases the rate of competitor-resistant complex formation (by at least 20-fold).\(^{52}\) This effect occurs primarily by accelerating isomerization of the complex (i.e., in the step after initial promoter binding). In addition, a substitution in $\alpha$CTD that prevents its DNA binding reduces the overall rate of complex formation by at least 20-fold.\(^{53}\) Therefore, it was reasonable to hypothesize that the sequence determinants responsible for DNA wrapping might be required for the large (20-fold) effect on isomerization.

To determine whether specific sequences within the upstream regions affect the rate of formation of RNAP promoter complexes at $P_R$, the overall second-order association rate constants ($k_a$) and rates of isomerization ($k_2$) were determined for the formation of heparin-stable complexes with fragments containing wt $P_R$ or three of the upstream substituted promoters (−79, −59, and −35; Fig. 5).

The association rate constants ($k_a$) and isomerization rate constants ($k_2$) for these four $P_R$ fragments differed by less than 2-fold (Fig. 5b). The values observed ($k_a=6.9 \times 10^5$ M$^{-1}$ s$^{-1}$; $k_2=2.3 \times 10^{-2}$ s$^{-1}$ for the full-length $P_R$ fragment) were in good agreement with the values previously determined for $P_R$ under very similar solution and temperature conditions (20 °C; $k_a=6.4 \times 10^5$ M$^{-1}$ s$^{-1}$; $k_2=1.4 \times 10^{-2}$ s$^{-1}$).\(^{54}\) We conclude that the upstream sequence determinants that correlate with DNA wrapping (Fig. 3) do not increase the rate of RNAP association with $P_R$, since the nonspecific sequence substituted for native $\lambda$ sequence in these promoter constructs (Fig. 1a) was functionally equivalent to the native $\lambda$ sequence in its effects on association rate. Rather, we suggest that sequence-nonspecific interactions withRNAP account for the previously observed effects of upstream DNA on the rate of association of RNAP with $P_R$. These findings are consistent with the observation that the presence of upstream DNA also affects the isomerization rate at $\lambda$CTV5,\(^{23}\) a promoter that does not contain an UP element\(^{25}\) and for which we have previously shown that it does not wrap upstream DNA.\(^{15}\) Our results are also consistent with the previous finding that substitution of native $P_R$ sequence upstream of −60 did not affect the kinetics of promoter complex formation (cited in Davis et al.\(^{22}\)).
**P<sub>RM</sub> occupancy in the context of a P<sub>R</sub>‐bound RNAP**

The DNA contour length measurements presented above indicate that RNAP interacts extensively with the upstream regions of both P<sub>R</sub> and P<sub>RM</sub>. For instance, an RNAP bound at P<sub>R</sub> is in contact with DNA beyond the start site of P<sub>RM</sub>, whereas an RNAP bound at P<sub>RM</sub> is in contact with the DNA region surrounding the −35 element of P<sub>R</sub>. As a result of this geometry, simultaneous binding of RNAP to P<sub>R</sub> and P<sub>RM</sub> should be difficult. However, previous studies have shown that binding to P<sub>R</sub> and P<sub>RM</sub> is not mutually exclusive, although binding to P<sub>RM</sub> is much slower in the context of an RNAP bound to P<sub>R</sub>.<sup>5,7</sup> In order to quantitatively determine the fraction of DNA templates in which both promoters are effectively occupied by an RNAP, AFM images were analyzed by counting the number of complexes comprising one or two RNAPs. DNA templates in which both P<sub>R</sub> and P<sub>RM</sub> are occupied by an RNAP are easily discernible by AFM because they are characterized by two adjacent globular features located near the center of the template (Fig. 6). It must be noted that, from the AFM images, it is not possible to distinguish whether the RNAP bound to either P<sub>R</sub> or P<sub>RM</sub> forms transcriptionally competent RPos. The results are summarized in Table 2, which shows that, in the case of RPos assembled with a DNA template harboring wt P<sub>R</sub> and wt P<sub>RM</sub>, 45% of the total DNA molecules seen microscopically had at least one RNAP bound. Of those, 98% had one RNAP, and 2% had two RNAPs (i.e., had both P<sub>R</sub> and P<sub>RM</sub> simultaneously occupied by RNAP). In controls, the same analysis was performed on complexes obtained with P<sub>R</sub> (−35 to +34) in which P<sub>RM</sub> had been deleted. In this case, 41% of the total molecules had at least one RNAP bound. Of those, 98% had one RNAP, and 2% had two RNAPs. Similarly, with the P<sub>R</sub>−wt P<sub>RM</sub> DNA template, which should form RPos only at P<sub>RM</sub>, we observed that 25% of the total molecules had at least one RNAP bound, 98% of which had one RNAP and 2% of which had two RNAPs. Thus, it appears that the small fraction of double complexes observed with these DNA templates represents the background of the measurements. On the other hand, in the case of P<sub>R</sub> (−79 to +34) for which a reduced DNA compaction of RPos formed at P<sub>R</sub> was observed, the double complexes were 8% of the total number of complexes scored. Consistently, RPos assembled with RNAP αCTD mutants on the wt P<sub>R</sub>−wt P<sub>RM</sub> DNA template, which are characterized by a reduced DNA compaction relative to wt RNAP (these data were extracted from AFM images relative to experiments published in Cellai et al.¹⁵), the percentage of double complexes was increased compared to that of the wt RNAP. Thus, it appears that, under our experimental con-

### Table 2. Frequency of DNA templates with one or two RNAPs bound at P<sub>R</sub>−P<sub>RM</sub>

<table>
<thead>
<tr>
<th>RNAP</th>
<th>DNA template</th>
<th>Complexes with at least one RNAP (%)</th>
<th>Complexes with two RNAPs (%)</th>
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<tbody>
<tr>
<td>wt RNAP</td>
<td>P&lt;sub&gt;R&lt;/sub&gt;−wt P&lt;sub&gt;RM&lt;/sub&gt;</td>
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<td>wt RNAP</td>
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<td>2</td>
</tr>
<tr>
<td>wt RNAP</td>
<td>P&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>wt RNAP</td>
<td>P&lt;sub&gt;R&lt;/sub&gt; (−79 to +34)</td>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td>Δ6-α&lt;sup&gt;+&lt;/sup&gt;/Δ6-α&lt;sup&gt;−&lt;/sup&gt; RNAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P&lt;sub&gt;R&lt;/sub&gt;−wt P&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Δ12-α&lt;sup&gt;−&lt;/sup&gt;/Δ12-α&lt;sup&gt;−&lt;/sup&gt; RNAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P&lt;sub&gt;R&lt;/sub&gt;−wt P&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>ΔαCTD&lt;sup&gt;+&lt;/sup&gt; RNAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P&lt;sub&gt;R&lt;/sub&gt;−wt P&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>71</td>
<td>3</td>
</tr>
<tr>
<td>ΔαCTD&lt;sup&gt;−&lt;/sup&gt;/ΔαCTD&lt;sup&gt;−&lt;/sup&gt; RNAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P&lt;sub&gt;R&lt;/sub&gt;−wt P&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>24</td>
<td>4</td>
</tr>
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</table>

Double complexes are those in which two RNAPs are bound to the same DNA template and their position is compatible with binding to P<sub>R</sub> and P<sub>RM</sub>. An example of such complexes is shown in Fig. 6. The percentage of single complexes is given with respect to the total number of molecules seen microscopically (free DNA, single RPos, and double RPos), whereas the percentage of double complexes has been determined with respect to the total number of complexes (single RPos plus double RPos). Δ6-α<sup>+</sup>/Δ6-α<sup>−</sup> RNAP and Δ12-α<sup>−</sup>/Δ12-α<sup>−</sup> RNAP are RNAP derivatives lacking 6 (α-residues 235–241) and 12 (α-residues 235–247) amino acid residues of the α-linker, respectively. ΔαCTD<sup>−</sup> RNAP is an RNAP derivative lacking both αCTD and αCTD<sup>D</sup>. ΔαCTD<sup>D</sup> / ΔαCTD<sup>D</sup> RNAP is an RNAP derivative lacking both αCTD and αCTD<sup>D</sup>.<sup>a</sup> Data extracted from AFM images of experiments published in Cellai et al.¹⁵
conditions, binding to $P_{RM}$ is more efficient when wrapping of the DNA around the $P_R$-bound RNAP is compromised either by changes in the upstream region of $P_R$ or by αCTD mutations.

**Discussion**

In this study, we have demonstrated that the DNA compaction that results when RNAP binds the $\lambda_{P_R}$ promoter to form an RPo can be accounted for by a direct interaction of RNAP with upstream DNA sequence determinants: one located in the region from $-80$ to $-100$, and the other located in the region from $-36$ to $-59$. Both regions contain ATR sequences that are similar to those shown to interact with RNAP αCTD.\(^{18,27,28}\) In a previously published AFM study,\(^{15}\) we have shown that the DNA compaction observed at $P_R$ is significantly reduced when the complexes are assembled with RNAP αCTD mutants, consistent with the hypothesis that αCTD mediates the RNAP–DNA interaction with the upstream promoter region. Because of the size of the polymerase molecule ($\sim 10$ nm in diameter)\(^{29}\) and the 100-bp contour length of DNA involved in the interaction ($\sim 34$ nm), DNA wrapping around the protein surface must be invoked to account for the hypothesized upstream protein–DNA interaction that results in DNA compaction.\(^{14,16,30,31}\) The relationship between observed compaction and modeled wrapping is schematically shown in Fig. 7a.

In the case of wt $P_R$–wt $P_{RM}$, contact between the RNAP and sequences in the region $-80$ to $-100$ of $P_R$ results in an almost complete turn of DNA around the polymerase, with consequent arrangement of $P_{RM}$ core promoter on the surface of the $P_R$-bound RNAP (Fig. 7b). Substitution of the sequence upstream of region $-79$ of $P_R$ with heterologous DNA eliminates the stretch of thymines from positions $-91$ to $-100$. This substitution results in a disassembled DNA compaction [20±0.5 nm for $P_R$ ($-79$ to $+34$) against $30±0.3$ nm for wt $P_R$–wt $P_{RM}$], which, in turn, suggests partial unwrapping of the DNA from the RNAP (Fig. 7c). Moreover, substitution of the sequence upstream of $-35$ resulted in a further reduction of the DNA compaction observed at $P_R$ [4±0.7 nm for $P_R$ ($-35$ to $+34$) against $30±0.3$ nm for wt $P_R$–wt $P_{RM}$], indicating that the ATR found within this region is also specifically contacted by an RNAP bound at $P_R$. Such a small DNA compaction, most probably due to the fact that the RNAP holds part of the DNA out of the surface as depicted in Fig. 7a, suggests that the RNAP–DNA interaction is limited to the $-10$ and $-35$ hexamers of $P_R$, with no upstream DNA wrapping involved (Fig. 7d). The DNA contour length analysis of RPo formed at the $P_{RM}$ promoter shows a DNA compaction of 18 nm, which reduces to 8 nm upon substitution of the ATR between regions $-40$ and $-60$ of $P_{RM}$, suggesting that this ATR is specifically contacted by the RNAP when bound to $P_{RM}$.

**Significance of RNAP–DNA upstream interactions**

What are the implications of the stable upstream interactions made by the RNAP at $P_R$ and $P_{RM}$? Previous work has shown that A-tracts are good αCTD binding sites and can increase the activity of the promoter.\(^{16,27,28}\) In addition, a recent study has demonstrated that the presence of upstream DNA increases the rate of RPo formation at $P_R$ by a factor of 35 and 60, at 37 and 17 °C, respectively.\(^{29}\) Thus, we hypothesized that the DNA wrapping determined by the interaction of the RNAP with upstream ATRs might have the same effect on the isomerization rate also because a change in writhe is physically linked to a change in twist. Contrary to our hypothesis, the data suggest that although $P_R$ ATRs are responsible for the extended RNAP–DNA interaction that structurally results in DNA wrapping, they have no stimulatory effect on the activity of $P_R$ in vitro; rather, we have found a small inhibitory effect of these upstream regions that may originate from steps after RPo formation. This result is in agreement with previous work showing that sequence-specific interactions involving $P_R$ and $P_{RM}$ ATRs have little effect on the activity of these promoters in vitro.\(^{20,22}\) We thus conclude that although αCTD upstream DNA contacts are important for promoting the formation of the RPo (upstream DNA increases the rate of competitor-resistant complex formation by at least 20-fold),\(^{22}\) this contact may be transient and does not require specific A-tract sequences that are needed for wrapping.

In the particular context of the divergent promoters $P_R$ and $P_{RM}$ which control the synthesis of the antagonist regulators $cI$ and $cro$, DNA wrapping becomes particularly interesting because it involves sequences that overlap with the divergent promoter and thus may suggest a mechanism for mutual interference between these promoters. Our finding that an RNAP bound at $P_R$ contacts sequences beyond the start site of $P_{RM}$ indicates that the $-10$ and $-35$ hexamers of the latter must lie on the surface of the $P_R$-bound RNAP, a geometry that should reduce the accessibility of $P_{RM}$. In keeping with this hypothesis is the observation that, when RPos were formed with wt template and wt RNAP, the fraction of complexes displaying both promoters occupied by polymerases was very small. Conversely, when RPos were formed with the mutated αCTD RNAPs, the fraction of double complexes was significantly increased (Table 2), in accordance with the smaller DNA compaction established for these mutants that should uncover the $P_{RM}$'s recognition sites. A similar result was also obtained with a DNA template ($P_R$ $-79$ to $+34$) in which the ATR between $-80$ and $-100$ was substituted for heterologous DNA while maintaining $P_{RM}$. This substitution seems to be sufficient for an RNAP to gain access to $P_{RM}$ in the context of an RNAP bound at $P_R$. The correlation between loss of wrapping and the frequency of double complexes in both of these experiments suggests the possibility of a causal relationship. It is also possible that alternative mecha-
nisms could contribute to the frequency of double complexes. For example, the RNAP α-subunit mutations could potentially affect this frequency by reducing the rate of formation of complexes at Pₐ. However, effects on the rate of Pₐ complex formation would not account for the increase in double complex frequency with the −79 to +34 promoter fragment, since little or no effect on the rate of Pₐ complex formation was observed with this fragment (Fig. 5).

Previous work has shown that, in the case of phage λ, an RNAP can bind to P RM also in the context of a P₀-bound RNAP, but it is unable to form a transcriptionally competent complex. The data presented herein indicate that simultaneous binding to Pₐ and P RM is rare in the wt case, and it increases whenever DNA wrapping at Pₐ is impaired. A possible explanation of such a discrepancy may reside in the different experimental conditions. In particular, for AFM experiments, RPs were formed with a 1:1 RNAP/DNA ratio (higher ratios complicate image interpretation because of many free RNAP molecules on mica support), whereas previous abortive initiation, gel shift, and DNA footprinting assays were performed with a large excess of RNAP with respect to the promoter DNA. Furthermore, it must be pointed out that in Hershberger and deHaseth and Hershberger et al., experiments were performed with an up-mutation of P RM to facilitate in vitro transcription assays and which may account for the increased P RM occupancy. Different salt concentrations and pH

Fig. 7 (legend on next page)
values might also have an effect on the probability of promoter binding.

Finally, two studies have shown that deletions between the ~35 elements of P_R and P_RM surprisingly reduce interference between these promoters: 12,13 This effect was more pronounced for the D10 construct in which a 10-bp deletion reduced the spacer between the ~35 elements to 2 bp. As suggested, the 10-bp deletion may relieve interference by placing the two promoters in an ideal configuration with their respective contacts with RNAP. We further hypothesize that a deletion within this region directly affects P_R and P_RM ATRs and changes the spatial relation of the upstream sequence determinants with respect to the RNAP αCTDs. This may affect the DNA-wrapping ability of P_R and P_RM. An AFM analysis of transcription complexes formed with the D10 would be required to better understand this unexpected behavior.

Based on previous and present data, our working hypothesis on the mechanism of promoter interference between P_R and P_RM can be summarized as follows: an RNAP quickly binds to the strong promoter P_R, wraps the DNA up to position ~100, and forms an open complex. Because of the αCTD interaction with upstream ATRs, DNA wrapping is stable and constrains the P_R promoter elements on the surface of the P_R-bound RNAP. Under these circumstances, binding to P_RM is still possible; however, in order to be accomplished, the DNA has to be unwrapped from the RNAP bound at P_R and this unwrapping can constitute an additional rate-limiting step during open complex formation at P_RM. Furthermore, gaining access to the ~10 and ~35 elements of P_RM in the context of a P_R-bound RNAP may not be sufficient for complete open complex formation at P_RM because of the lack of free upstream DNA known to accelerate isomerization. 22,23 The absence of upstream DNA contacts might contribute to slowed open complex formation at P_RM.

Fig. 7. Model of the proposed upstream DNA interaction in the RPo formed at P_R. (a) Schematic representation showing the relationship between the DNA compaction observed by AFM and modeled DNA wrapping. In the absence of wrapping, an RNAP (blue circle) can adhere to mica in an orientation such that the DNA (thick purple line) is held above the surface plane. The contour length of the DNA line is 6.1d, where d is the diameter of the RNAP (d ~ 10 nm). Assuming that all the complexes are deposited as depicted in the leftmost drawing, the apparent DNA contour length (r) measured from the top view (thick dashed line in the middle drawing) is 5.5d. Thus, this arrangement produces a compaction of 0.6d (~6 nm). In a more realistic situation, surface-bound complexes are randomly oriented; thus, we expect an average DNA compaction of 4 nm or less in the absence of wrapping. In the case of an almost complete turn of DNA wrapping (right drawing), DNA contour length measurements are also affected by the tip broadening effect (outlined by the gray dashed circle), as a result of which the DNA in proximity to the RNAP is hidden from the observer. Therefore, during contour length measurements, the DNA path is drawn through the center of the protein, as shown by the thick dashed line, which has a length of 3.5d and a resulting compaction of 2.6d (~26 nm). If we consider that part of the wrapped DNA can also be held out of the mica plane, the expected DNA compaction for such an arrangement is approximately 3d (~30 nm), in good agreement with the value experimentally measured. (b) With a wt DNA template, an RNAP bound at P_R wraps the DNA by contacting AT-rich sequences up to position ~100 (Fig. 1a). With this geometry, the P_SAP promoter lies on the surface of the RNAP, presumably in proximity to the αCTD. (c) Substitution of the DNA sequence between ~80 and ~100 eliminates an ATR region, causing partial loss of DNA wrapping with a consequent increased accessibility to P_R. (d) Substitution of the DNA sequence upstream of ~35 eliminates two ATRs (one in the region between ~80 and ~100, and the other in the region between ~40 and ~60) and completely abolishes DNA wrapping at P_R. Polymerase has been drawn based on the structure determined by Murakami et al. 32 (Protein Data Bank ID 1L9Z). RNAP β- and β′-subunits are in light and dark blue, respectively; α-subunits are in light and dark green; and α-subunit is in yellow. The αCTD, which is absent in the crystal structure, has been drawn to scale as green ellipsoids. A schematic representation of the DNA is drawn to scale in light and dark purple.

Materials and Methods

DNA

The 1054-bp-long DNA template wt P_R−wt P_RM was obtained by HindIII digestion of plasmid pSAP. 14 This template contains λ-DNA from ~438 to +34 with respect to the P_R start site, which is positioned at 616 bp from the downstream end. The 975-bp-long DNA template P_R (~100 to +34) was obtained by PCR from plasmid pPR100 using primers NEB_FOR (5’-AAAAACCTCTGACACATG- CAGC) and NEB_REV (5’-GCTGCCCCCTTTGCTCA- CATG). pPR100 was constructed by cloning the pSAP sequence from ~100 to +100 into the Smal restriction site of pNEB193 (New England Biolabs). The 954-bp-long DNA template P_R (~79 to +34) was obtained by PCR from plasmid pPR79 using primers NEB_FOR and NEB_REV. pPR79 was constructed by cloning the pSAP sequence from ~79 to +100 into the Smal and HindIII restrictions sites of pNEB193. The 963-bp-long DNA template P_R (~59 to +34) was obtained by PCR from plasmid pPR59 using primers NEB_FOR and NEB_REV. pPR59 was constructed by cloning the pSAP sequence from ~59 to +100 into the HindIII restriction site of pNEB193. The 963-bp-long DNA template P_R (~35 to +34) was obtained by PCR from plasmid pPR35 using primers 5’-AAAAACCTCTGACA- CATGCAGC and 5’-GCTGCCCCCTTTGCTCAATG. pPR35 was constructed as follows: a 160-bp DNA fragment was obtained by PCR from pSAP using a primer designed to amplify the pSAP region from ~35 to ~100. The forward primer used in this PCR was obtained as such to insert 24 bp of heterologous DNA upstream of position ~35, with respect to PR, in order to maintain in registry the sequence of the different constructs. The resulting 160-bp insert was cloned into the HindIII restriction site of pNEB193.

The 1003-bp-long DNA template P_R−wt P_RM was obtained by PCR from plasmid pPRM using primers 5’-TGGAAATACCTTCACACCACG and 5’-GCTGCTT- GGCTCAAGC CGG. pPRM is a pSAP derivative in which the ~10 hexamer of P_R has been inactivated as described in Tang et al. 20 The 999-bp-long DNA template P_RM (~35 to +541) was obtained by PCR from plasmid
pPRM35 using primers NEB_FOR and NEB_REV. pPRM35 was constructed by cloning the λ sequence from -35 to +79 with respect to PRM into the HincII restriction site of pNEB193. In analogy to pPR35, the reverse primer used to amplify the λ sequence was obtained as such to insert 24 bp of heterologous DNA upstream of position -35 of PM.

PCR amplification was carried out in standard reaction conditions using Deep Vent DNA polymerase. Preparative restriction digestes were carried out overnight. All DNA fragments were purified on a 1% (wt/vol) agarose gel and recovered by electro elution in an Elutrap apparatus (Schleicher & Schuell, Keene, NH). The DNA was extracted with phenol–chloroform, precipitated with ethanol, and resuspended in TE buffer (50 mM Tris–HCl (pH 7.4) and 1 mM ethylenediaminetetraacetic acid). The DNA concentration was determined by absorbance at 260 nm. PCR amplification of promoter fragments for in vitro transcription and association rate measurements is described below.

**RNA polymerases**

Wild-type *E. coli* RNA polymerase in AFM experiments and runoff transcription assays was prepared as described in Estrada et al. or purchased from Epicenter Biotechnologies (Madison, WI). Wild-type *E. coli* RNA polymerase used in association kinetic measurements was purified as described in Burgess and Hendriks, and determined to be ~60% active, as described in Ross and Gourse.

**RPo formation and AFM imaging**

RPo's were prepared by mixing 20 fmol of DNA template and 20 fmol of RNA polymerase in transcription buffer [20 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and 1 mM DTT]. The 10-μl reaction was incubated at 37 °C for 20 or 40 min. The reaction was diluted to 1–2 nM complexes in 20 μl of deposition buffer [4 mM Hepes (pH 7.4), 10 mM NaCl, and 4 mM MgCl₂] and immediately deposited onto freshly cleaved ruby mica (Mica, New York, NY). The sample was incubated for about 2 min before the surface was rinsed with water milli-Q (Millipore) and dried with a weak stream of nitrogen. AFM imaging was performed on the dried sample with a Nanoscope III microscope (Digital Instruments, Inc., Santa Barbara, CA) operating in tapping mode. Commercial diving board silicon cantilevers (Nanosensor or Olympus) were used. Images of 512×512 pixels were collected with a scan size of 2 μm at a scan rate of three to four lines per second.

**Runoff transcription assays**

Runoff transcription assays were performed under conditions similar to those used in the AFM experiments. Linear DNA fragments of ~400 bp were obtained by PCR from the constructs described above. DNA fragments were such that, in all cases, the runoff transcripts initiated at Pₐ would have a length of 101 nt, whereas transcripts initiated at PₐM would have lengths as follows: wt Pₐ−wt Pₐ, 154 nt; 2×Pₐ−wt Pₐ, 160 to +34, 154 nt; 2×Pₐ−PₐM, 140 nt; Pt − +34, 140 nt; Pt − +34, 140 nt; Pₐ−wt PₐM, 154 nt; and PₐM−PₐM, 137 nt. DNA and RNAP at a final concentration of 20 nM each were mixed in transcription buffer, and the reaction was incubated at 37 °C for 40 min to allow open complex formation. Subsequently, heparin (to a final concentration of 100 μg/ml) was added to the reaction, followed by the addition of 10 U of SUPERase-In (Ambion) as RNase inhibitor, 10 μCi of [α-32P]UTP (Amersham Biosciences), and nucleoside triphosphates to a final concentration of 20 μM each. Transcription was allowed to proceed for 15 min at room temperature. The 10-μl reactions were terminated by dipping the tubes into dried ice and by adding 30 μl of gel loading buffer. The reactions were heated at 90 °C for 2 min and loaded onto a 6% polyacrylamide gel and 7 M urea gel. The gel was scanned with a Personal Imager FX (Bio-Rad). Bands corresponding to transcription products were then quantified using the MultiAnalyser PC software (Bio-Rad).

**Association kinetic measurements for RNAP binding to λPR**

Linear fragments of similar lengths (~165 bp; promoter sequence from -115 to +50) were prepared by PCR amplification from each of the four plasmids: pSP (wt Pₐ−wt PₐM), pPR79 (Pₐ (−79 to +34)), pPR89 (Pₐ (−59 to +34)), and pPR35 (Pₐ (−35 to +34)). Forward primers were either 5′-GTACGAAATTCGATCCACGATACCATGATACAATCCACACGTATGATGTAATCTTTCTTTTGTCCTC for pSP. The reverse primer was 5′-CAGGACCGGGAACGTTTTTAAATACACTTATACATTCTCCTGTTTACACCGT for all plasmids. PCR products were purified using QIAquick PCR Purification columns, and 5 pmol of each was digested with HindIII (encoded in the reverse primer at position +50 with respect to the transcription start site). Nontemplate strand HindIII site 3′ ends were labeled by filling in with [α-32P]dATP (Perkin Elmer) and Sequenase (US Biochemicals), and labeled fragments were digested with EcoRV (encoded in forward primers) to create a blunt upstream end at position -115. Labeled fragments were gel-purified, eluted, and concentrated using Ultrapip columns (Whatman).

Rates of association of RNAP to form heparin-resistant complexes were determined by a filter binding assay, essentially as described previously. Under these conditions, binding to λPR is much faster than binding to λPRM, and only complexes at λPR are detected. Briefly, fragments (~1 nM) were incubated with a series of concentrations of wt RNAP (with RNAP in at least 3 μM) for 5 min in a buffer fragment containing 0.15 M Tris–HCl (pH 8.0), 120 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 100 μg/ml bovine serum albumin. The RNAP preparation was used ~60% active, as determined by a promoter titration assay (see Ross and Gourse), and values in Fig. 5 were determined using concentrations of active RNAP. Reactions, carried out at 20 °C, were initiated by addition of RNAP. At time intervals, aliquots were sampled to tubes containing heparin (final concentration, 50 μg/ml) and filtered after 30 s. Radioactivity retained on filters was quantified by exposure to a phosphorimager screen. Data for each active RNAP concentration were plotted as counts per minute retained versus time, and an observed rate constant, kₐobs, was determined from fitting to the equation: 

$$k_{a, obs} = \frac{[\text{cpm}_{obs} - \text{cpm}_{plateau}]}{(1 - e^{-t})}$$

Data in Fig. 5a are shown in a τ plot (1/kₐobs versus 1/[RNAP]). Values in Fig. 5b were determined from nonlinear plots of kₐobs versus [RNAP]. Data were fitted to the equation 

$$k_{a, obs} = k_{a} [\text{RNAP}] (k_{b} [\text{RNAP}] + k_{3})$$

To determine the composite association rate constant kₐ and the isomerization rate constant k₃ for each fragment, k₁ was determined from the relationship k₁ = kₐk₃. The kₐ and k₃ values for the wt fragment were consistent with values
previously reported for λpR under these temperature and salt conditions.24

Image analysis

AFM images were analyzed using a software written in MATLAB environment. DNA contour length measurements were performed as described in Rivetti and Codeluppi.23 DNA molecules suited for analysis were selected by visual inspection based on the following criteria: the molecule was completely visible on the image, its contour was not ambiguous, RNAP was bound at the expected position, and no other proteins were bound to the same DNA. Data were elaborated with MATLAB and graphed with Sigmaplot (Systat Software, Inc., California). Contour length distributions were fitted with a Gaussian function using Sigmaplot.

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References


