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Sequence-dependent upstream DNA-RNA polymerase interactions in the open complex with λP_R λP_{RM} promoters and implications for the mechanism of promoter interference

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Abstract

The upstream interactions of *Escherichia coli* RNA polymerase in open complex (R_{Po}) formed at the P_R and P_{RM} promoters of bacteriophage lambda, have been studied by atomic force microscopy (AFM). We demonstrate that the previously described 30 nm DNA compaction observed upon R_{Po} formation at P_R ¹ 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, R_{Po}s formed at P_{RM} showed a specific contact between the RNAP and the DNA sequence from –36 to –60. We further demonstrate that this interaction, which results in DNA wrapping against the polymerase surface, is mediated by the C-terminal domains of the alpha subunits (α CTD). Substitution of these AT-rich sequences with heterologous DNA reduces DNA wrapping but has little 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 α CTD 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.

Keywords

Transcription; DNA wrapping; promoter interference; upstream ATR; atomic force microscopy AFM

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Introduction

The bacteriophage λ promoters P_R and P_{RM} control the expression of repressor proteins *cro* and *cI* which drive the establishment of the lytic and lysogenic states². P_R and P_{RM} are divergently transcribed from start sites separated by a 82 base pair (bp) sequence harboring the -10 and -35 hexamers of both promoters and the operator sites O_R1 , O_R2 and O_R3 ³. Differential binding of *cI* 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^{4,5}. Open complex (R_{Po}) formation at P_R is much faster than open complex formation at P_{RM} , thus, binding of a RNA polymerase (RNAP) to P_{RM} only takes place in the context of an RNAP bound to P_R ^{6,7}. It has been shown that P_{RM} activity is increased by mutations designed to inactivate P_R ^{8,9,10,11} and conversely, an RNAP bound to P_{RM} interferes with the utilization of a weakened P_R promoter¹². 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 the isomerization from closed complex to open complex^{8,9,10,11}. Contrary to the expectation, a ten 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 a relief of interference^{13,14}. Studies aimed to elucidate the structure of the λ P_R open complex have shown that, at variance with other promoters, the DNA is tightly wrapped around the RNAP extending the RNAP-DNA interaction further upstream than the P_R -35 element^{1,15,16}. This extended contact is generally mediated by the α -subunits carboxy-terminal domains (α CTD) that interact with (A+T)-rich DNA sequences (ATR) located upstream of the promoter^{17,18,19}. 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 open promoter complexes formed at P_R , 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 R_{Po} 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 R_{Po} formed at this promoter, but also the previously described P_R - P_{RM} promoter interference.

Results

To assess the involvement of the ATR regions positioned upstream of P_R in the DNA compaction of R_{Po} we have constructed P_R promoter variants in which the upstream DNA was selectively substituted with heterologous DNA and measured the DNA compaction of the corresponding R_{Po}. Figure 1a reports the DNA sequence of the promoter variants used in this study and Figure 1b shows representative AFM images of R_{Po}. R_{Po} 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, 1 mM DTT) at 37 °C for at least 20 minutes. R_{Po} activity was verified by run-off transcription assays conducted under similar conditions but with the

addition of heparin to prevent multiple rounds of transcription (Figure 2). Heparin could not be used in the AFM experiments because it prevents adhesion of the DNA to the mica support, thus the RPo seen microscopically were considered such because they were formed under conditions that favour open complex formation and were located at the promoter site.

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

The 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 (Figure 2, lane 1) and by previous work^{6,7,8,11}. Therefore, complexes assembled with this DNA template were considered as RPo formed at P_R . The DNA contour length analysis of these complexes shows an average DNA compaction of 30 nm (Figure 3a and Table 1) in full agreement with previously published data^{1,15}. A DNA compaction of 30 nm was also observed with P_R (−100 to +34) (Figure 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 it is not contacted by the RNAP.

Conversely, RPo formed with P_R (−79 to +34), in which the DNA sequence upstream of −79 was substituted with vector DNA, show a DNA compaction of 20 nm (Figure 3c and Table 1). This result indicates that in the region from −80 to −100 of P_R there must be a sequence determinant which is specifically contacted by an RNAP bound at P_R . Because a DNA compaction of 20 nm is still significantly high compared to other promoters¹⁵, we hypothesized that other sequence determinants capable of interacting with the RNAP must be present in the upstream sequence of P_R . RPo formed with P_R (−59 to +34), in which the DNA sequence upstream of −59 was substituted with vector DNA, show again a DNA compaction of 20 nm (Figure 3d and Table 1). Thus, the DNA region from −60 to −79 does not contain sequence determinants involved in DNA compaction. Substitution of the DNA sequence upstream of −35, as in the case of P_R (−35 to +34), resulted in a DNA compaction of only 4 nm. Such a little DNA compaction indicates 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 region from −36 to −59 of P_R .

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

similarity, we hypothesized that the RNAP-DNA interaction at P_{RM} should involve DNA sequences in the region from -36 to -60 of P_{RM} .

In order to verify this hypothesis, we constructed P_{RM} (-35 to $+541$), a λP_{RM} derivative in which the sequence upstream of -35 has been replaced with heterologous DNA (Figure 1a; the P_R promoter -10 and -35 hexamer regions are deleted in this construct). The contour length analysis of these complexes is shown in Figure 4b and Table 1. RPo complexes at P_{RM} (-35 to $+541$) display a DNA compaction of 8 nm thus confirming the presence of a sequence determinant in the upstream region of P_{RM} which 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 (Figure 1a) affect the functional interaction of RNA polymerase 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 (Figure 2, lanes 1,2). However, substitutions for upstream regions closer to the P_R promoter resulted in small increases in P_R expression (~ 1.5 to 2.5-fold greater than wt for the -79 , -59 and -35 substitutions; Figure 2, lanes 3-5). These results suggest the possibility of a small inhibitory effect of the wild-type upstream regions on expression from P_R . The observed increases in transcription did not correlate with overall rates of RNAP association with these promoters (k_a ; see below), suggesting that the mechanism of the increases may involve steps after RNAP-promoter complex formation. Consistent with this possibility, inhibitory effects of A+T-rich upstream sequences on promoter clearance have previously been noted for promoters that are rate-limited at this step²¹.

Transcription from the P_{RM} promoter was not detected for any of the promoter region fragments that contain a P_R promoter (including, as expected, for fragments -79 , -59 and -35 , in which all or part of P_{RM} was deleted; Figure 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 Figure 1a; lane 6 in Figure 2). The substitution for sequences upstream of the -35 region of P_{RM} ($P_{RM}(-35$ to $+541)$, Figure 1a) removed the P_R promoter and resulted in a small increase in P_{RM} transcription (<2 -fold; Figure 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 λP_R fragments containing different lengths of upstream DNA (a “full length” fragment with upstream DNA to -110 and an upstream truncated fragment with upstream DNA only 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)²². This effect occurs primarily by accelerating isomerization of the complex (i.e., on a step after initial promoter binding). In addition, a substitution in

the α CTD that prevents its DNA binding reduces the overall rate of complex formation by at least 20-fold²³. 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 sequences within specific 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 formation of heparin stable complexes with fragments containing wt P_R or three of the upstream substituted promoters (−79, −59 and −35; Figure 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 (Figure 5b). The values observed ($k_a = 6.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$; $k_2 = 2.3 \times 10^{-2} \text{ s}^{-1}$ for the full length P_R fragment) were in good agreement with values previously determined for P_R under very similar solution and temperature conditions (20 °C; $k_a = 6.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$; $k_2 = 1.4 \times 10^{-2} \text{ s}^{-1}$)²⁴. Thus, we conclude that the upstream sequences that correlate with DNA wrapping (Figure 3) do not play a specific role in the rate of RNAP association with P_R . Rather, we conclude that the nonspecific sequence that was substituted for native lambda sequence in these promoter constructs (Figure 1a) is functionally equivalent to the native lambda sequence in its effects on association rate. These findings are consistent with the observation that the presence of upstream DNA also affects the isomerization rate at *lacUV5*²³, a promoter that does not contain an UP element²⁵, and for which we have previously shown that it does not wrap upstream DNA¹⁵. 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 ref. ²²).

P_{RM} occupancy in the context of a P_R -bound RNAP

The DNA contour length measurements presented above, indicate that RNAP interacts extensively with the upstream region of both P_R and P_{RM} . For instance, an RNAP bound at P_R is in contact with DNA beyond the start site of P_{RM} whereas an RNAP bound at P_{RM} is in contact with the DNA region surrounding the −35 element of P_R . As a result of this geometry simultaneous binding of RNAP to P_R and P_{RM} should be difficult. However previous studies have shown that binding to P_R and P_{RM} is not mutually exclusive although binding to P_{RM} is much slower in the context of an RNAP bound to P_R ^{6,8}. 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 comprised of one or two RNA polymerases. DNA templates in which both P_R and P_{RM} 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 (Figure 6). It must be noted that from the AFM images it is not possible to distinguish whether the RNAP bound to either P_R or P_{RM} forms transcriptionally competent open promoter complexes. The results are summarized in Table 2 which shows that in the case of RPo assembled with a DNA template harboring wt P_R and wt P_{RM} , 44% of the total DNA molecules seen microscopically had one RNAP bound at the promoter and 2% of these complexes had both P_R and P_{RM} simultaneously occupied by an RNAP. As a control, the same analysis was performed on complexes obtained with P_R (−35 to +34) in which P_{RM}

has been deleted. In this case 40% of the total molecules were single complexes, 2% of which were double complexes. Similarly, with the P_R^- – wt P_{RM} DNA template, which should form RPo only at P_{RM} , we observed that 24% of the total molecules were single complexes and 2% of these were double complexes. 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_R (–79 to +34), for which a reduced DNA compaction of RPo formed at P_R was observed, the double complexes were 9% of the total number of complexes scored. Consistently, RPo assembled with RNAP- α CTD mutants on the wt P_R – wt P_{RM} 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 ref. ¹⁵), the percentage of double complexes was increased compared to the wt RNAP. Thus, it appears that under our experimental 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 α CTD mutations. However, we cannot exclude the possibility that the DNA substitutions or the RNAP α -subunit substitutions may affect the frequency of doubly-occupied promoter fragments by altering the relative rates of promoter complex formation at the two promoters, independent of, or in addition to, the proposed effects of wrapping. For instance, substitutions or deletions of α CTD reduce the rate of association of RNAP with both P_R and P_{RM} ^{20,23} and it is therefore possible that the lack of α CTD-DNA upstream interactions, when using a mutant RNAP, would differentially affect the rates of complex formation at the two promoters, thereby favoring a greater level of P_{RM} occupancy. In addition, although there are no sequence-specific elements in the downstream region of promoters that are known to affect open complex formation or stability, it is a formal possibility that the substitution in the P_{RM} promoter downstream region might affect the frequency of simultaneous occupancy of P_{RM} and P_R with the P_R (–79 to +34) promoter fragment.

Discussion

In this study we have demonstrated that the DNA compaction that results when RNAP binds the λP_R promoter to form an open promoter complex can be accounted for by 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 α CTD^{18,26,27}. In a previously published AFM study¹⁵, we have shown that the DNA compaction observed at P_R is significantly reduced when the complexes are assembled with RNAP- α CTD mutants, consistent with the hypothesis that α CTD mediate the RNAP-DNA interaction with the upstream promoter region. Because of the size of the polymerase molecule (~10 nm in diameter)²⁸ and the contour length of 100 bp of DNA involved in the interaction (~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^{1,16,29,30} (Figure 7a).

Substitution of the sequence upstream of –79 of P_R with heterologous DNA, eliminates the stretch of thymines from positions –91 to –100. This substitution results in a diminished DNA compaction (20 ± 0.5 nm for P_R (–79 to +34) against the 30 ± 0.3 nm for wt P_R – wt P_{RM}) which, in turn, suggests a partial unwrapping of the DNA from the RNAP (Figure 7b).

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 the 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 suggests that the RNAP-DNA interaction is limited to the -10 and -35 hexamers of P_R , with no upstream DNA wrapping involved (Figure 7c). 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 -40 and -60 of P_{RM} , suggesting that this ATR is specifically contacted by the RNAP when bound to P_{RM} .

Significance of the 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 α CTD binding sites and can increase the activity of the promoter^{18,26,27}. 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 °C and 17 °C respectively²². 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 which 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} 's ATRs have little effect on the activity of these promoters *in vitro*^{20,25}. We thus conclude that although α CTD-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 the 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 the 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 RPo were formed with the wild-type 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 α CTD-RNA polymerases the fraction of double complexes was significantly increased (Table 2), in accordance with the smaller DNA compaction established for these mutants which should uncover the P_{RM} 's recognition sites. A similar result was also obtained with DNA templates in which one of the two ATRs were deleted while maintaining P_{RM} . In particular, deletion of the ATR between -80 and -100 seems to be sufficient for an RNAP to gain access to P_{RM} in the context of an RNAP bound at P_R . However, since this deletion removes P_{RM} sequences downstream of

–3, it is also possible that the substituted sequences make P_{RM} stronger so that it can form stable complexes in spite of an RNAP bound to P_R .

Previous work has shown that in the case of phage λ , an RNAP can bind to P_{RM} also in the context of a P_R -bound RNAP but it is unable to form a transcriptionally competent complex^{8,9,10,11}. The data presented herein, indicate that simultaneous binding to P_R and P_{RM} is rare in the wild-type case, and it increases whenever DNA wrapping at P_R is impaired. A possible explanation of such a discrepancy may reside in the different experimental conditions. In particular, for AFM experiments, RPo were formed with a 1:1 RNAP:DNA ratio (higher ratios complicate the image interpretation because of the many free RNAP molecules on the 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 ref. ⁸ and ⁹ 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 concentration and pH 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 the interference between these promoters^{13,14}. This effect was more pronounced for the D10 construct in which a deletion of 10 bp 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 α CTDs. 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 about 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 α CTD interaction with upstream ATRs, DNA wrapping is stable and constrains the P_{RM} promoter elements on the surface of the P_R -bound RNAP. Under these circumstances, binding to P_{RM} is still possible but 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 at the –10 and –35 elements of P_{RM} in the context of a P_R -bound RNAP, may not be sufficient to complete open complex formation at P_{RM} because of the lack of free upstream DNA that is known to accelerate isomerization^{22,23}. The absence of upstream DNA contacts might contribute to slowed down open complex formation at P_{RM} .

Materials and Methods

DNA

The 1054 bp long DNA template wt P_R – wt P_{RM} , was obtained by Hind III digestion of plasmid pSAP¹. 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'-AAAACCTCTGACACATGCAGC) and NEB_REV (5'-GCTGCCCTTTTGCTCACATG). pPR100 was constructed by cloning the pSAP sequence from –100 to +100 into the SmaI 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 SmaI and HindIII restrictions site 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 HincII 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'-AAAACCTCTGACACATGCAGC and 5'-GCTGCCCTTTTGCTCACATG. pPR35 was constructed as follows: a 160 bp DNA fragment was obtained by PCR from pSAP using primer designed to amplify the pSAP region from –35 to +100. The forward primer used in this PCR reaction was such to insert upstream of position –35 with respect to P_R , 24 bp of heterologous DNA in order to maintain in register the sequence of the different construct. The resulting 160 bp insert was cloned into the HincII 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'-TGGAATTACCTTCAACCTCAAGC and 5'-CTGTTTTGGTCTAAGCTGCGG. pPRM is a pSAP derivative in which the –10 hexamer of P_R has been inactivated as described in²⁰. 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 +137 with respect to P_{RM} into the HincII restriction site of pNEB193. In analogy to pPR35, the primer reversed used to amplify the λ sequence was such to insert upstream of position –35 of P_{RM} , 24 bp of heterologous DNA.

PCR amplification was carried out in standard reaction conditions using Deep Vent DNA polymerase. Preparative restriction digests were carried out overnight. All DNA fragments were purified on 1% (w/v) agarose gel and recovered by electro elution in an Elutrap apparatus (Schleicher & Schuell, Keene NH). The DNA was phenol-chloroform extracted, ethanol precipitated and resuspended in TE buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA). The concentration of the DNA was determined by absorbance at 260 nm.

RNA Polymerases

Wild-type E. coli RNAP used in AFM experiments and run-off transcription assays was prepared as described in¹⁸ or purchased from Epicentre Biotechnologies (Madison, WI).

Wild-type *E. coli* RNAP used in association kinetic measurements was purified as described in ref. ³¹, and was determined to be ~60% active as described in ref. ²³.

RPo formation and AFM imaging

RPo complexes were prepared by mixing 20 fmol of DNA template and 20 fmol of RNAP in transcription buffer (20 mM Tris-HCl pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT). The 10 µl reaction was incubated at 37 °C for 20 or 40 minutes. The reaction was diluted to 1-2 nM complexes in 20 µl of deposition buffer (4 mM Hepes pH 7.4, 10 mM NaCl, 4 mM MgCl₂) and immediately deposited onto freshly-cleaved ruby mica (Mica New York, NY). The sample was incubated for about two minutes 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 3-4 lines per second.

Run-off transcription assays

Run-off transcription assays were performed under conditions similar to 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 run-off transcripts initiated at P_R would have a length of 101 nt, whereas transcripts initiated at P_{RM} would have a length as follows: wt P_R – wt P_{RM} 154 nt, P_R (–100 to +34) 154 nt, P_R (–79 to +34) 140 nt, P_R (–59 to +34) 140 nt, P_R (–35 to +34) 140 nt, P_R[–] – wt P_{RM} 154 nt and P_{RM}(–35 to + 541) 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 minutes to allow open complex formation. Subsequently, heparin to a final concentration of 100 mg/ml was added to the reaction followed by the addition of 10U SUPERase-In (Ambion) as an RNase inhibitor, 10 µCi [α-³²P]UTP (Amersham Biosciences), and NTPs to a final concentration of 20 µM each. Transcription was allowed to proceed for 15 minutes at RT. The 10 µl reactions were terminated by dipping the tubes into dried ice and by addition of 30 µl of gel loading buffer. The reactions were heated at 90 °C for 2 minutes and loaded onto a 6% polyacrilamide, 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 MultiAnalyst PC software (Bio-Rad).

Association Kinetic Measurements for RNAP binding to λP_R

Linear fragments of similar length (~165bp; promoter sequence from –115 to +50) were prepared by PCR amplification from each of four plasmids: pSAP (wt P_R – wt P_{RM}), pPR79 (P_R (–79 to +34)), pPR59 (P_R (–59 to +34)) and pPR35 (P_R (–35 to +34)). Forward primers were either: 5'-GTACGAATTCGATATCCAGCTATGACCATGATTACGCCAAGC for plasmids pPR79, pPR59 and pPR35, or 5'-GTACGAATTCGATATCTGTGTTAATGGTTTCTTTTTTGTGCTC for pSAP. The reverse primer was: 5'-CAGGACCCGGAAGCTTTTAATTAACACTCTTATACATTATTCC 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). Non-template strand HindIII site 3' ends were labeled by filling in with [α - 32 P]-dATP (Perkin Elmer) and Sequenase (US Biochemicals), and labeled fragments were digested with EcoRV (encoded in the forward primers) to create a blunt upstream end at position -115. Labeled fragments were gel purified, eluted and concentrated using ElutipD columns (Whatman).

Rates of association of RNAP to form heparin resistant complexes were determined by a filter binding assay, essentially as described^{7,23}. Under these conditions, binding to λP_R is much faster than to λP_{RM} , and only complexes at λP_R are detected⁷. Briefly, fragments (<1 nM) were incubated with a series of concentrations of wt RNAP (with RNAP in at least 3X molar excess over fragment) in a buffer containing 10 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 100 μ g/ml bovine serum albumin. The RNAP preparation used was ~60% active as determined by a promoter titration assay (see ref. ²³), and values in Figure 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 of 50 μ g/ml), and after 30 seconds were filtered. Radioactivity retained on filters was quantified by exposure to a phosphorimager screen. Data for each active RNAP concentration was plotted as CPM retained vs time, and an observed rate constant, k_{obs} , was determined from fitting to the equation: $[cpm_{obs} = (cpm_{plateau})(1 - e^{-k_{obs}t})]$. Data in Figure 5a are shown in a τ plot ($1/k_{obs}$ vs. $1/[RNAP]$). Values in Figure 5b were determined from nonlinear plots of k_{obs} vs. $[RNAP]$. Data were fit to the equation: $k_{obs} = k_a k_2 [RNAP] / (k_a [RNAP] + k_2)$ to determine the composite association rate constant k_a and the isomerization rate constant k_2 for each fragment. K_1 was determined from the relationship $k_a = k_2 K_1$. Values for k_a and k_2 for the wild-type fragment were consistent with values previously reported for λP_R under these conditions of temperature and salt concentration²⁴.

Image analysis

AFM images were analyzed using software written in the Matlab environment. DNA contour length measurements were performed as described in³². DNA molecules suited for analysis were selected by visual inspection based on the following criteria: The molecule had to be completely visible in the image, its contour was not ambiguous, the RNAP was bound at the expected position, no other proteins were bound to the same DNA. Data were elaborated with Matlab and graphed with Sigmaplot (Systat Software, Inc. CA). Contour length distributions were fitted with a Gaussian function using Sigmaplot.

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Abbreviations used

AFM	atomic force microscopy
RNAP	RNA polymerase
RPo	open promoter complex
CTD	carboxy-terminal domain

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Figure 1.

a) Sequences of upstream regions of λP_R and λP_{RM} variants used in this work. The start site +1, the -10 and -35 hexamers are singly underlined for P_R and doubly underlined for P_{RM} . AT-rich regions (ATR) 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 RPo complexes formed with wt RNAP and the wt P_R – wt P_{RM} DNA template. c) AFM image of RPo complexes formed with αCTD^{II} RNAP and the wt P_R – wt P_{RM} DNA template. The image scan size is 2 μm .

Figure 2.

Single round *in vitro* transcription of the DNA templates shown in Figure 1. Lanes: 1) wt P_R – wt P_{RM} ; 2) P_R (–100 to +34); 3) P_R (–79 to +34); 4) P_R (–59 to +34); 5) P_R (–35 to +34); 6) P_R^- – wt P_{RM} ; 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 an inhomogeneous run-off termination. The P_{RM} transcripts in lanes 6 and 7 have different length because of the different length 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.

Figure 3.

DNA contour length distributions of bare DNA and RPo formed with wt RNAP and P_R derivatives as follows: a) $wt P_R - wt P_{RM}$; b) $P_R (-100 \text{ to } +34)$; c) $P_R (-79 \text{ to } +34)$; d) $P_R (-59 \text{ to } +34)$; e) $P_R (-35 \text{ to } +34)$. In all panels, white-dashed bars represent bare DNA contour length frequencies, dark-gray bars represent RPo contour length frequencies. The solid lines is the Gaussian fitting of the distributions. Mean and SE values derived from the fitting are reported in Table 1. The DNA compaction, defined as the difference between mean values of DNA and RPo distributions, is reported on each graph.

Figure 4.

DNA contour length distributions of bare DNA and RPo at the P_{RM} promoter. a) RPo formed with wt RNAP and the $P_R^- - wt P_{RM}$ DNA construct. b) RPo formed with wt RNAP and the $P_{RM} (-35 \text{ to } +541)$ DNA construct. White-dashed bars represent bare DNA contour length frequencies, dark-gray bars represent RPo contour length frequencies. The solid lines are the Gaussian fitting of the distributions. Mean and SE values derived from the fitting are reported in Table 1. The DNA compaction is reported on each graph.

a

b

Promoter	$k_a \times 10^{-5}$ ($M^{-1}s^{-1}$)	$k_2 \times 10^2$ (s^{-1})	$1/k_2$ (sec)	$K_1 \times 10^{-7}$ (M^{-1})
wt P _R – wt P _{RM}	6.9 ± 0.9	2.3 ± 0.9	43	3.0
P _R (-79 to +34)	4.2 ± 0.6	2.8 ± 1.8	36	1.5
P _R (-59 to +34)	6.7 ± 1.4	3.6 ± 2.5	28	1.9
P _R (-35 to +34)	4.3 ± 0.9	1.6 ± 0.8	63	2.7

Figure 5.

Effects of upstream substitutions on association of wt RNAP with the λP_R promoter. a) Double-reciprocal (τ) plots (see Materials and Methods) from reaction performed in a range of RNAP concentrations with 165 bp DNA fragments (from –115 to +50 of P_R) at 20 °C. wt P_R – wt P_{RM} (filled circles), P_R (–79 to +34) (open triangles), P_R (–59 to +34) (open diamonds), P_R (–35 to +34) (filled squares). b) Kinetic constants derived from the data shown in a.

Figure 6.

Top and lateral views of a DNA template in which P_R and P_{RM} are both bound to and RNAP molecule. Because of the geometry of the DNA template, the two complexes can not be distinguished. These double complexes were rare in the case of wt P_R and wt P_{RM} . However, their frequency increased when DNA wrapping at P_R was impaired by mutations either in the upstream DNA or in the RNAP α -subunits.

Figure 7.

Model of the proposed upstream DNA interaction in the open promoter complex formed at P_R . a) With a wt DNA template, an RNAP bound at P_R wraps the DNA by contacting AT-rich sequences up to position -100 (Figure 1a). With this geometry, the P_{RM} promoter lies on the surface of the RNAP, presumably in proximity of the α -CTD. b) Substitution of the DNA sequence between -80 and -100 eliminates an ATR, causing a partial loss of DNA wrapping with a consequent increased accessibility of P_{RM} . c) 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 . The polymerase has been drawn based on the structure determined by the laboratory of S. Darst³³ (pdb id: 1L9Z). The RNAP β and β' subunits are in light and dark blue, the α subunits are in light and dark green and the σ subunit is in yellow. The α -CTD, absent in the crystal structure, have been drawn to scale as green ellipsoids. A schematic representation of the DNA is drawn to scale in light and dark purple.

Table 1

Summary of the bare DNA and RPo contour length measurements performed in this work.

RNAP	DNA Template	Promoter	DNA length (bp)	Bare DNA contour length (nm)	RPo contour length (nm)	DNA compaction (nm)
wt-RNAP	wt P _R – wt P _{RM}	P _R	1054	338 ± 0.2 (N=157)	308 ± 0.2 (N=353)	30 ± 0.3
wt-RNAP	P _R (–100 to +34)	P _R	975	315 ± 0.2 (N=155)	285 ± 0.3 (N=225)	30 ± 0.4
wt-RNAP	P _R (–79 to +34)	P _R	954	312 ± 0.2 (N=92)	292 ± 0.4 (N=117)	20 ± 0.5
wt-RNAP	P _R (–59 to +34)	P _R	963	313 ± 0.3 (N=143)	293 ± 0.4 (N=90)	20 ± 0.5
wt-RNAP	P _R (–35 to +34)	P _R	963	315 ± 0.4 (N=102)	311 ± 0.5 (N=138)	4 ± 0.7
wt-RNAP	P _R [–] – wt P _{RM}	P _{RM}	1003	325 ± 0.4 (N=115)	307 ± 0.5 (N=68)	18 ± 0.6
wt-RNAP	P _{RM} (–35 to +541)	P _{RM}	999	323 ± 0.6 (N=70)	315 ± 0.7 (N=76)	8 ± 0.9

Contour length values of bare DNA and RPo represent the mean ± SE obtained from the fitting of the DNA contour length distributions shown in figure 3 and 4. N is the number of molecules measured. The DNA compaction is given by the difference between the mean DNA contour length of bare DNA and that of RPo.

Table 2

Frequency of DNA templates with single and double complexes.

RNAP	DNA Template	Percent of single complexes	Percent of double complexes
wt-RNAP	wt P _R – wt P _{RM}	44 %	2 %
wt-RNAP	P _R (–35 to +34)	40 %	2 %
wt-RNAP	P _R [–] – wt P _{RM}	24 %	2 %
wt-RNAP	P _R (–79 to +34)	38 %	9 %
6-α ^I / 6-α ^{II} RNAP [*]	wt P _R – wt P _{RM}	10 %	10 %
12-α ^I / 12-α ^{II} RNAP [*]	wt P _R – wt P _{RM}	10 %	10 %
αCTD ^{II} RNAP [*]	wt P _R – wt P _{RM}	25 %	3 %
αCTD ^I / αCTD ^{II} RNAP [*]	wt P _R – wt P _{RM}	65 %	4 %

Double complexes are those in which two RNA polymerases are bound to the same DNA template and their position is compatible with binding to P_R and P_{RM}. An example of such complexes is shown in Figure 6. The percentage of single complexes is given with respect to the total number of DNA molecules whereas the percentage of double complexes has been determined with respect to the total number of single RPo. 6-α^I/ 6-α^{II} RNAP and 12-α^I/ 12-α^{II} RNAP are RNAP derivatives lacking six (α-residues 235–241) and twelve (α-residues 235–247) amino-acid residues of the α-linker respectively³⁴. αCTD^{II} RNAP is an RNAP derivative having αCTD^I but lacking αCTD^{II}18. αCTD^I/ αCTD^{II} RNAP is an RNAP derivative lacking both αCTD^I and αCTD^{II}18.

^{*} Data extracted from AFM images of experiments published in¹⁵.