Promoter Recognition by Escherichia coli RNA Polymerase: Effects of the UP Element on Open Complex Formation and Promoter Clearance †

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Promoter Recognition by *Escherichia coli* RNA Polymerase: Effects of the UP Element on Open Complex Formation and Promoter Clearance†

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ABSTRACT: *Escherichia coli* promoters for transcription of ribosomal and tRNAs are greatly activated by an A+T-rich "UP" element upstream of the −35 region. These same promoters have also been found to otherwise deviate in several respects from the consensus promoter sequence. Here we present the results of a kinetic characterization of the interaction of *Escherichia coli* RNA polymerase with UP element-containing promoters which by virtue of consensus or near-consensus sequence features should be among the most optimal that can be encountered by *Escherichia coli* RNA polymerase. We show that for such promoters, (1) the second-order rate constant describing formation of the initial (closed) complex is close to that expected for a diffusion-limited process, (2) the extent of activation by the UP element is temperature-sensitive, (3) the UP element accelerates a process after DNA binding by RNA polymerase, and (4) the presence of the UP element delays promoter clearance upon addition of nucleoside triphosphates to preformed RNA polymerase−promoter complexes. Finally, we provide evidence in support of models which describe the DNA melting process accompanying open complex formation as initiating in the −10 promoter region and progressing in the downstream direction.

The identity of promoters for *Escherichia coli* RNA polymerase (RNAP)[1] is chiefly determined by two DNA elements, the −10 and −35 regions (*1*); these two elements, together with the spacer DNA separating them and the start site of transcription, form the so-called "core" promoter. The ability to recognize the core promoter is imparted to the RNAP holoenzyme by its sigma subunit. The promoters for transcription of ribosomal RNA (*2*), as well as some other strong promoters in *E. coli* (*3*−*6*) and *B. subtilis* (*7*, *8*), additionally have a very (A+T)-rich region upstream of base pair −40 (the so-called "UP" element). Transcription in vivo and in vitro as well as the rate of open complex formation at the *rrnB* P1 promoter (*9*) was decreased by more than 10-fold upon deletion of this UP element. The UP element has also been shown to activate promoters that do not naturally contain such an element (*2*, *9*, *10*).

Ribosomal RNA promoters can direct initiation of one transcript per second, which is more than any other promoter characterized to date. The paradigm of UP element containing promoters, rrnB P1, deviates from the sequence of the consensus core promoter in several ways, including the sequence of the −35 element, the length of the spacer DNA separating the −35 and −10 regions, and the sequence of the DNA region that becomes strand-separated upon formation of the functional or open RNAP−promoter complex (*2*, *9*). As a result of these features, open complexes formed in vitro at this and other ribosomal RNA promoters are unstable (*11*), but can be stabilized in the presence of high concentrations of the initiating nucleoside triphosphate (*12*). Open and closed complexes are in rapid equilibrium, in which the closed form of the promoter predominates, but addition of nucleoside triphosphates can pull the equilibrium dramatically in the direction of the opened form. Using NTP stabilization of open complexes at ribosomal RNA promoters, it has been determined that open complex formation in vitro is very fast. Kinetic investigations on these promoters by necessity have been carried out under conditions of very low concentrations of RNAP and promoter in order to bring the rates into experimentally tractable time regimes.

We have undertaken a kinetic characterization of promoters which by virtue of their UP element, in combination with entirely consensus −10 and −35 regions, should be among the fastest to form open complexes with *E. coli* RNAP. These studies exploited a recently developed spectroscopic assay for RNAP−promoter complexes which allows the determination of the kinetics of formation of these complexes over the time scale of seconds (*13*). We show that the second-order rate constant describing formation of the initial (closed) complex is close to that expected for a diffusion-limited process, that the extent of activation by the UP element is temperature-sensitive, that the UP element accelerates a process after DNA binding by RNAP, and that the presence of the UP element delays promoter clearance upon addition of nucleoside triphosphates to preformed RNAP−promoter complexes. We also provide further experimental support for a model where nucleation of DNA melting initiates in the −10 region and propagates in the downstream direction.

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[1] Abbreviations: RNAP, RNA polymerase; oligo, oligodeoxyribonucleotide; 2-AP, 2 aminopurine.

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MATERIALS AND METHODS

Materials. E. coli RNAP holoenzyme prepared by the method of Burgess and Jendrisak (*14*) was further purified by chromatography on phosphocellulose in the presence of 50% glycerol, as described (*15*). The concentrations of RNAP reported here are those of the active holoenzyme; our preparations were 50 ± 10% active. 2-AP-containing oligos were purchased from core facilities at the University of Wisconsin, Madison, or at Case Western Reserve University; other synthetic DNAs were from Gibco/BRL. Promoters with 2-AP substitutions were assembled by ligation of synthetic DNA oligos. Proper assembly was monitored by running analytical agarose gels. Purification of assembled promoters was accomplished by the use of 10% nondenaturing polyacrylamide gels. Ribonucleoside triphosphates were purchased from Pharmacia and used without further purification.

Stopped-Flow Experiments. Open complex formation at 2-AP-substituted promoters was studied in transcription buffer [30 mM HEPES (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, and 10% glycerol] on an Applied Photophysics SX18MV stopped-flow spectrophotometer in the fluorescence mode as described (*13*). The excitation monochromator slits were set at 2 nm, and the wavelength at 315 nm. A 350 nm "cut on" filter (transparent to light with a wavelength greater than 350 nm) was used for the emitted light. Each determination of k_{obs} involved averaging of data collected in three or more mixing events. The promoter DNA (5 nM) and the RNAP solutions loaded in the syringes of the instrument were adjusted to contain the same concentration (10%) of glycerol in order to obtain optimal mixing.

Data Analysis. Initial data analysis was carried out with the software provided with the stopped flow. The experiments were conducted with RNAP in at least 3-fold excess over the promoter fragments so that the kinetics would be pseudo-first-order. The data were fit to the equation:

$$y = \mathrm{Amp}[1 - \exp(-k_{obs}t)] + F_o \qquad (1)$$

Here y is the observed fluorescence signal at 370 nm, t is the time in seconds after mixing promoter DNA and RNAP, k_{obs} is the pseudo-first-order rate constant, Amp is the observed amplitude (i.e., the total change in signal between $t = 0$ and $t = \infty$), and F_o is the starting fluorescence signal. For each promoter, k_{obs} was obtained at 25 and 10 °C as a function of RNAP concentration. The parameter k_f (see eqs 3 and 4 under Results) as well as the second-order rate constant k_a was obtained from the hyperbolic fit of the data to the equation:

$$k_{obs} = k_a k_f[\mathrm{RNAP}]/(k_a[\mathrm{RNAP}] + k_f) \qquad (2)$$

Promoter Clearance Assays. Promoter clearance experiments were carried out at room temperature (23 °C) on a Perkin-Elmer LS50B spectrofluorometer using a 300 μL micro cell. Samples were excited at 310 nm (5 nm slit width), and emission was monitored at 370 nm (15 nm slit width). The reaction was initiated by the addition of 75 μL of an NTP/heparin mixture to a cuvette containing 225 μL of a 1:1 mixture of RNAP and promoter DNA (incubated for an hour to ensure complete formation of RNA polymerase−promoter complex at even the weakest promoters investigated here) to yield 300 μL of a solution containing 10 nM promoter complex, 200 μM of each NTP, and 50 μg/mL heparin. The fluorescence intensity was monitored over a time span of 10 min. Each clearance result is based on the average of the data from at least 10 separate determinations. The averaged data were fit to a single exponential:

$$y = \mathrm{Amp}[1 - \exp(-k_{clr}t)] + F_o$$

where y, Amp and F_o are as in eq 1, and k_{clr} is the first-order clearing constant. In Figure 3, values on the y-axis represent $(y - F_o)$/Amp, to obtain normalized curves.

RESULTS

The two consensus promoter constructs (designated UP^+-CC and UP^-CC to indicate whether the UP element sequence is present or not, and that the −35 and −10, respectively, are consensus) used in this work are shown in Figure 1. The UP element is from rrnB P1, the spacer from P_{RM}, as is the start site and the region downstream from it. Other constructs differ with respect to sequences of the −35 (N = 5′TAGATA3′) and −10 (N = 5′TAGAAT3′) regions; e.g., UP^+NC has the UP element, with −35 = 5′TAGATA3′ and −10 = 5′TATAAT3′. Fast kinetic experiments were carried out by mixing solutions of RNAP and 2-AP-substituted promoter DNA on a stopped-flow spectrophotometer (*13*). The observed increase in 2-AP fluorescence, primarily due to unstacking of (2-AP) bases in the region melted by RNA polymerase, was followed over time intervals of 1−10 s, depending on the promoter being studied. Observed rates of open complex formation were determined by fitting the time dependence of the 2-AP-derived fluorescence signal to a single exponential, as described [(*13*); Materials and Methods]. These values are shown as a function of the concentration of RNAP in Figures 2a,b. For the CC promoter, the observed rate constants are among the fastest obtained for an *E. coli* promoter. The presence of the UP element accelerates complex formation less than 2-fold at 25 °C, over the range of RNAP concentrations examined. This is in contrast to the experiments carried out both on the same promoter at 15 °C and on the nonconsensus promoter at 15 as well as 25 °C, when stimulation by the UP element is readily apparent at the higher concentrations of RNAP investigated. The lines drawn in Figures 2a,b represent hyperbolic fits (eq 2) of the data. Values of the parameters k_a and k_f are collected in Table 1.

The interpretation of the parameters k_a and k_f is model-dependent. Two different models will be considered, differing in whether the initial complex between RNAP and the promoter (RP_{c1}, where the subscript c denotes a closed complex where strand separation has not yet occurred) is in rapid equilibrium with free RNAP and promoter (eq 3), or is converted with high probability to the nucleated intermediate RP_{c2} (*16*, *17*), poised to proceed down the pathway to open complex formation (eq 4):

$$\mathrm{R + P} \overset{K_B}{\rightleftharpoons} \mathrm{RP_{c1}} \xrightarrow{k_2} \mathrm{RP_{c2}} \xrightarrow{\text{fast}} \mathrm{RP_o} \qquad (3)$$

$$\mathrm{R + P} \xrightarrow{k_1} \mathrm{RP_{c1}} \xrightarrow{k_2} \mathrm{RP_{c2}} \xrightarrow{\text{fast}} \mathrm{RP_o} \qquad (4)$$

Here R is RNAP, P is promoter, K_B is the association constant describing the rapid equilibrium between RNAP and

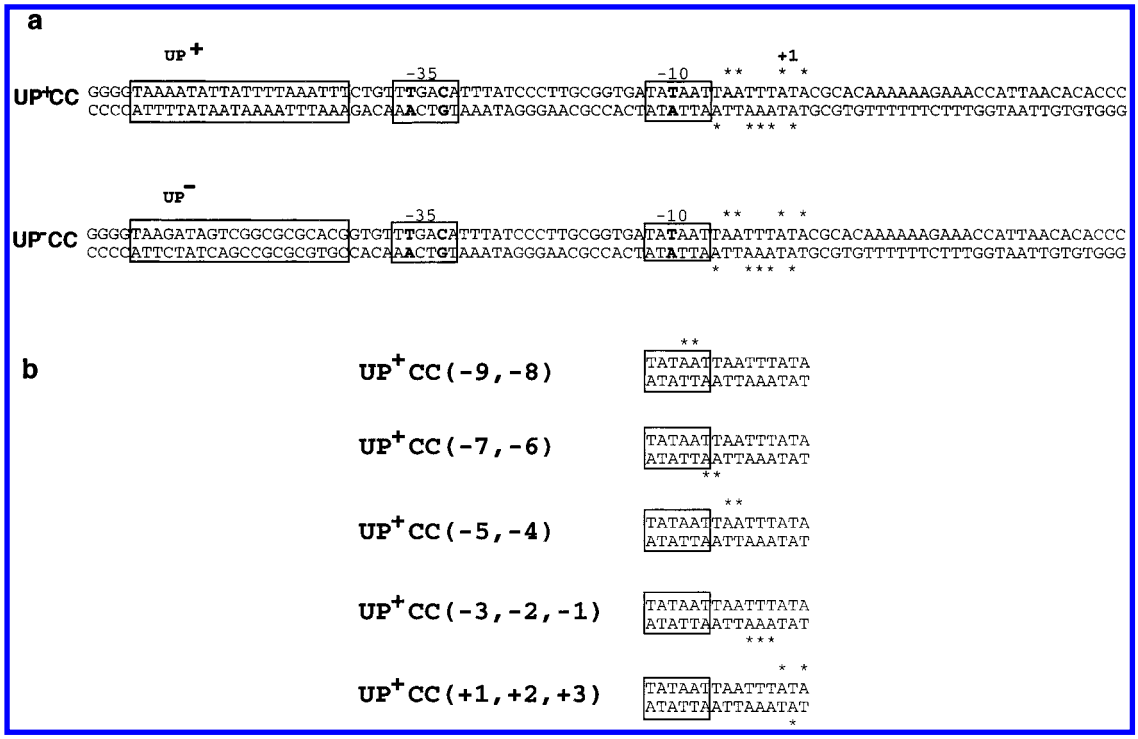

FIGURE 1: Sequences of synthetic promoter DNAs used in this work. Each promoter is 95 bp in length, spanning positions −58 to + 32 with respect to the start site. The −35 and −10 regions and the start site are indicated. (a) The top promoter (UP$^+$CC) has a functional UP element as indicated; the bottom one (UP$^-$CC) does not. The positions of the nine 2-AP (at −5, −4, +2, and +3 on the top strand, and −6, −3, −2, −1, and +2 on the bottom strand) are indicated by the asterisks. (b) The constructs with 2-AP substitutions (shown by asterisks) at different positions in the melted region.

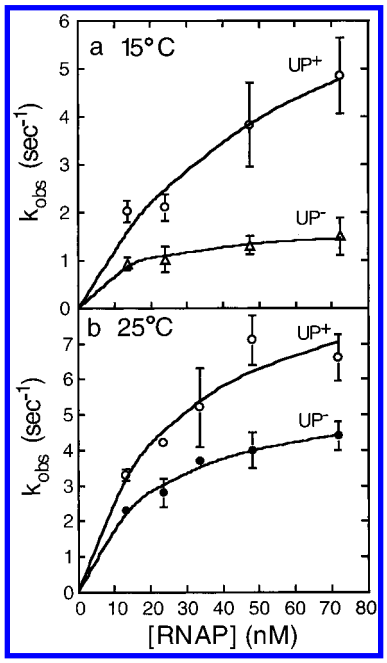

FIGURE 2: Effect of the UP element, for the CC promoter, at two different temperatures. The rate of open complex formation, k_{obs}, is plotted as a function of the concentration of RNAP. Each value of k_{obs} was obtained in at least three independent mixing events on the stopped-flow spectrophotometer. Error bars represent standard deviations determined from averaging these multiple determinations. The curves represent hyperbolic fits (eq 2) of the data. (a) Data obtained at 15 °C. (b) Data obtained at 25 °C.

the closed promoter complex, and RP_o is the open promoter complex. While the overall mechanism is likely more complex, this description of intermediates up to and including the rate-limiting step (the RP_{c1} to RP_{c2} conversion) is a useful way to treat kinetic data (*18*). The RP_{c1} to RP_{c2} step would be a conformational change in RNA polymerase; the actual DNA melting would occur in a subsequent step [see (*16*) and (*17*) for recent reviews]. For a reaction near the diffusion limit (as may be the case for all but the UP$^-$NC promoters), the appropriate description is eq 4; then $k_a = k_1$, and $k_f = k_2$ (*9*). The calculated values for the second-order rate constant, k_a, are among the largest observed for any promoter, and close to the expected diffusion-limited rate for the initial specific interaction of RNAP and a promoter, as required by eq 4. The effect of the UP element is found to be less pronounced at the strongest, CC, promoter, and at the higher temperature (25 °C). It can be seen from the data collected in Table 1 that the presence of an UP element, compared to the same promoter without an UP element, leads to increases in k_f ranging from 2-fold for CC at 25 °C to 8-fold for NC at 15 °C. The data in Table 1 also show that k_a is lowest for the NC promoter. Assuming that open complex formation at this slower promoter can be described by an initial rapid equilibrium (eq 3), then $k_a/k_f = K_B$ and again $k_f = k_2$. Values for K_B can be estimated from the data for UP$^-$NC: $K_B = k_a/k_f$ is $(5 \pm 1) \times 10^7$ M^{-1}, at both 15 and 25 °C.

Table 1: Kinetics of RNA Polymerase−Promoter Interaction: Effect of UP Element

promoter DNA	temp (°C)	k_f (s^{-1})	$k_a \times 10^{-8}$ (M^{-1} s^{-1})
UP$^+$CC	15	8.9 ± 2.7	1.4 ± 0.4
UP$^-$CC		1.7 ± 0.2	1.3 ± 0.3
UP$^+$CC	25	9.7 ± 1.7	3.6 ± 1
UP$^-$CC		5.7 ± 0.4	2.7 ± 0.4
UP$^+$NC	15	1.6 ± 0.1	0.7 ± 0.1
UP$^-$NC		0.2 ± 0.02	0.1 ± 0.01
UP$^+$NC	25	4.4 ± 1.3	1.1 ± 0.4
UP$^-$NC		1.0 ± 0.1	0.6 ± 0.1

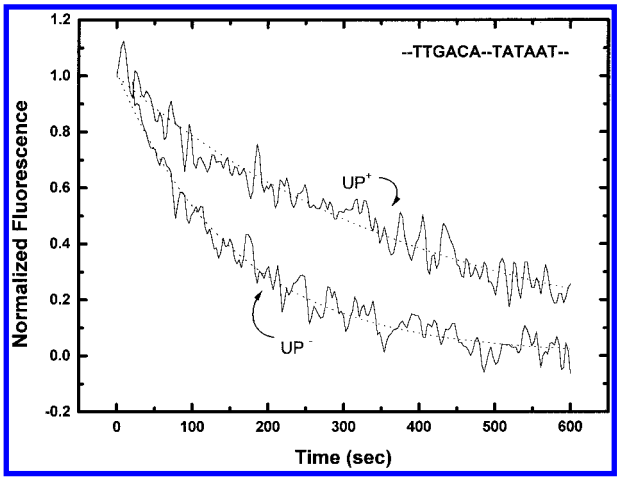

FIGURE 3: Effect of the UP element on promoter clearance. The time-dependence of the loss of fluorescent signal upon addition of NTPs to preformed open complexes (see text for experimental details) of the UP$^+$CC and UP$^-$CC promoters (Figure 1) at 25 °C is shown. Data collection was initiated following addition of a mixture of the four NTPs, each at 200 μM. Each trace is the average of at least seven determinations. The lines through the experimental traces are single-exponential fits. See Table 1 for the resulting rate constants.

Temperature-independent formation of RP_c had previously also been demonstrated for several other promoters. Based on the results presented above, we conclude that the UP element accelerates the conformational change in RNA polymerase that is the rate-limiting step in the formation of an open complex; as a result, another step, possibly DNA melting itself, may become rate limiting for promoters containing the UP element (see Discussion).

The above experiments demonstrate that the UP element plays a role during formation of the initiation-competent open complex. To determine if the effects of the UP element persist up to the time when RNAP clears the promoter to commence the elongation phase of RNA synthesis, rates of clearance were determined for the UP$^+$CC and UP$^-$CC promoters. This was done by monitoring the loss in fluorescence signal upon addition of nucleoside triphosphates, presumably as the result of reestablishment of base pairing in the promoter. We have found that this loss of signal is dependent on the addition of NTPs: in control experiments, no such loss of signal was observed over the time course of the experiment, if NTPs were not added (data not shown). In addition, we had previously shown that the presence of 2-AP substitutions at positions similar to those in UP$^+$CC and UP$^-$CC did not interfere with RNA synthesis (*13*). Thus, we are not simply determining the rate of dissociation of RNAP from promoters in these experiments. The results are shown in Figure 3, where the loss of fluorescence signal is displayed as a function of time after addition of the nucleoside triphosphates. At least 7 trials were carried out with each promoter. For the UP$^-$CC promoter shown in Figure 1, significantly faster rates of promoter clearance (observed rate constants as determined from single-exponential fits to the data; see Materials and Methods) were seen than for the promoter with an UP element, UP$^+$CC: The observed rate constants for promoter clearance (and the corresponding half-lives) have been collected in Table 2 for these as well as promoters containing nonconsensus substitutions in just the −10 region, or both the −10 and −35 regions, with or without the UP element. It is seen that the promoters without the UP element were cleared faster than those with the UP element by 1.5-fold for the NN and CN promoters, and by 3-fold for the CC promoter.

Table 2: Kinetics of Promoter Clearance: Effect of UP Element

promoter DNA	$k_{clr} \times 10^3$ (s^{-1})	$t_{1/2}$ (s)
UP$^+$CC	2.4 ± 0.2	290 ± 30
UP$^-$CC	6.4 ± 0.6	110 ± 10
UP$^+$CN	6.0 ± 0.1	116 ± 2
UP$^-$CN	9.3 ± 0.1	75 ± 1
UP$^+$NN	9.3 ± 0.1	76 ± 1
UP$^-$NN	15.0 ± 0.4	46 ± 1

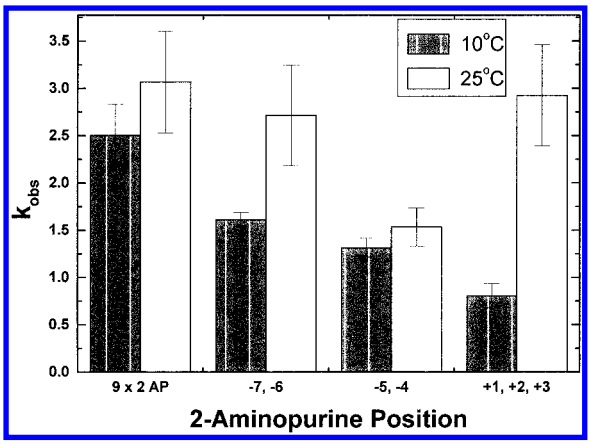

FIGURE 4: Effects of the positions of 2-AP substitutions in the UP$^+$CC promoter (see Figure 1) on the observed rate constants for open complex formation, k_{obs}. The locations of the 2-APs in each promoter DNA used are shown under the bar that represents the value for k_{obs} obtained for that promoter; see also Figure 1b. The concentrations of promoter DNA and RNAP were 15 and 50nM, respectively. Values for k_{obs} represent averages of 4−9 independent determinations, each itself the average of at least four individual traces. Error bars correspond to standard deviations in the averages. Grey bars: data obtained at 10 °C. White bars: data obtained at 25 °C.

With the constructs shown in Figure 1b, we have attempted to follow the progression of strand opening by introducing clusters of two or three 2-AP at various positions in the strand-separated region of the UP$^+$CC promoter. If propagation, rather than nucleation, of strand opening is rate limiting, 2-AP proximal to the nucleation site would be expected to open faster (i.e., with a greater k_{obs}) than more distal ones, giving rise to a gradient in observed rate constants. The results obtained with these constructs at a single RNAP concentration and temperatures of 10 and 25 °C are shown in Figure 4. For two promoters, UP$^+$CC(−9,−8), where the location of the 2-AP for A substitutions is shown by the numbers in parentheses, and UP$^+$CC(−3,−2,−1), no fluorescence signal was observed upon mixing of promoter DNA with RNAP, even if the binding reaction was monitored over 30 s (data not shown). This lack of signal does not reflect an inability to bind to RNAP. In gel mobility shift experiments (data not shown), we have determined that within 30 s of addition of RNAP (100 nM) to solutions containing 1 nM promoters UP$^+$CC(−9,−8) and UP$^+$CC(−3,−2,−1), essentially all promoter DNA was found in a heparin-resistant band with a mobility characteristic of an RNAP−promoter complex. Interestingly, we had also failed to obtain a fluorescence signal with single-stranded DNA containing the

2-AP substitutions at positions −9 and −8 (*19*). It is unclear what features of the interaction of the DNA with RNAP underlie the absence of an increase in the fluorescence signal when the 2-AP is at certain positions. For the constructs where a signal was observed, the rate constants determined at 10 °C were smallest if the 2-AP substitutions were furthest removed from the −10 region [in $UP^+CC(+1,+2,+3)$]. These observations are indicative of DNA melting being limiting, nucleation of the opening of promoter DNA occurring upstream of positions −6 and −7, and progression of strand separation being in the downstream direction. This course of events is consistent with current ideas about the progression of the strand separation process (*20*, *21*). At 25 °C, the construct $UP^+CC(+1,+2,+3)$ binds RNAP with an observed rate constant 3−4 times greater than that seen at 10 °C. The 25 °C data may correspond to a case where melting is not limiting; at this temperature, it may be the construct with the centrally located 2-AP [$UP^+CC(-5,-4)$], which, for unknown reasons, behaves anomalously. Experiments to further explore the behavior at 25 °C are in progress. Data obtained at 15 °C (not shown) show an intermediate effect: the ratio of k_{obs} for $UP^+CC(-7,-6)$ and $UP^+CC(+1,+2,+3)$ is 1.5, as compared to 2.0 at 10 °C, and 1.0 at 25 °C (see Figure 4).

DISCUSSION

We have observed remarkably high second-order rate constants for initial complex formation between RNAP and promoters, which for some constructs was close to 3×10^8 M^{-1} s^{-1}. No promoters have yet been observed to exhibit faster rates than those we have described here. As our promoters reside on very short DNA duplexes, this is an indication that a mechanism of rate enhancement for the binding of RNAP to its target, involving nonspecific DNA (e.g., linear diffusion or intersegment transfer), is not required to achieve fast rates of open complex formation. However, on long DNA molecules, some mechanism of transfer of RNAP from initially formed nonspecific complexes to promoter DNA may still be important, to overcome the adverse effects of nonspecific interactions in reducing the effective free RNAP concentration. Interestingly, the rates observed here for the formation of open complexes on promoter DNA are much faster than those observed under the same conditions for sequence-specific complex formation between RNAP and small, single stranded oligos ($k_a \sim 10^7$ M^{-1} s^{-1}). This difference may be an indication that promoter DNA is better able to elicit in RNAP a conformational change required for the formation of the open complex (*19*).

From the [RNAP] dependence of k_{obs} determined in experiments described here and also elsewhere (*10*), we conclude that the effect of the UP element is exerted at the level of a first-order process, after the binding of RNAP to the promoter, as reflected by the increase in k_f. Possibly the interaction of the alpha subunits of RNA polymerase with the UP element (*2*, *22*) facilitates a necessary conformational change in the RNA polymerase. The extent to which the presence of the UP element accelerated open complex formation was found to be a function of the sequence of the core promoter and the temperature at which the experiment was carried out. Greater effects were observed for the weaker promoters and in experiments performed at the lower temperature (see Figure 4 and Table 1). We speculate that more is to be gained by facilitating the conformational change under these conditions: both a greater similarity of the DNA sequence to the consensus −10 and −35 regions (as for the stronger promoters) and a higher temperature (*19*) may already facilitate the conformational change to some extent. If the UP element were to sufficiently accelerate the conformational change in RNA polymerase, under some conditions DNA melting might become limiting. As pointed out below, it is possible that this may be the case for the UP^+CC promoter at 10 °C. Recently a consensus sequence for the UP element was derived, which was found to be an even more potent activator than the rrnB P1 UP element (*23*). Its effect on the kinetics of open complex formation at a core promoter with consensus −10 and −35 sequences has not yet been determined.

It is important to address the extent to which the results obtained here may have been affected by the presence of the 2-AP substitutions in the promoter DNA. We had previously found that the presence of the same nine 2-AP substitutions as used here did not affect the rate of open complex formation (*13*) in a promoter similar to the UP^-NC one described here. The 2-AP:T base pair is compatible with the structure of double-helical DNA (*24*−*26*), but may be weaker than an A:T pair by about 0.5 kcal/mol (*27*), so that nine 2-AP base pairs may lower the stability of the melted region by as much as 5 kcal/mol. The lack of an observed effect of the 2-AP substitution, for the UP^-NC promoter, is thus consistent with prior observations [reviewed in (*16*) and (*17*)] that a conformational change in RNA polymerase rather than the DNA melting step is rate limiting for open complex formation.

Prior kinetic studies on the ribosomal RNA promoter, rrnB P1, are consistent with effects both on the bimolecular step (the first step in eq 3) and on a concentration-independent step (the second step in eq 3) (*9*), rather than just the latter as observed here. In comparing our promoter constructs containing a UP element to those lacking such an element, it is difficult to envision how the presence of the 2-AP substitutions could erroneously have lead to the conclusion that not DNA binding, but a subsequent process is accelerated by the UP element. A more likely possibility is that the mechanism whereby the UP element accelerates open complex formation is promoter dependent: It is possible that the UP element displays intrinsically different behavior in the context of the rrnB P1 promoter, at which unstable open complexes form (*11*), than upstream of promoters with which RNAP forms stable open complexes, such as those studied here. Our attempts to use 2-AP substituted DNA fragments bearing the rrnB P1 sequence in order to obtain a direct comparison thus far have been thwarted by the paucity of strategically positioned A's at which 2-AP substitutions can be made. As a result of a weak signal, we have not yet been able to collect reliable data.

For all but the UP^-NC promoter, we have assumed that k_a is the second-order rate constant for formation of the initial specific closed complex, RP_{c1}, at the promoter. The values for k_a we have determined here (as high as 3×10^8 M^{-1} s^{-1}; see Table 1) are in the range predicted for a diffusion-controlled process (*16*, *28*). However, relative values of k_a reflect relative rates of open complex formation only at concentrations of RNAP that are well below those needed to achieve a significant population of the initial complex.

At the limit of high concentrations of RNAP, rates of open complex formation are determined by k_f, which sets the levels of the plateaus for k_{obs}, as seen in the plots of Figure 2. Our attempts to determine the direction of DNA melting (shown in Figure 4) were made under these conditions, and employed the promoter UP$^+$CC, for which the conformational change in RNA polymerase would be optimally accelerated, possibly to the extent that DNA melting became rate limiting. For determinations carried out at 10 °C, a gradient in the values for k_{obs} is seen, with the values decreasing as the 2-APs are positioned in a more downstream direction of the promoter. We infer from these results that under these conditions melting may indeed be limiting, proceeding in a downstream direction, after being nucleated upstream of positions −6 and −7, possibly in the −10 region. The fact that clear position-dependent effects of 2-AP substitution were obtained makes it unlikely that the presence of the 2-AP substitutions themselves affected the outcome of these experiments. Thus, we feel that the interpretation of the results in terms of the relative rates of strand separation at various positions is valid. At 25 °C, no gradient in k_{obs} was found, which we interpreted to indicate that DNA melting was not limiting at this temperature. The construct which behaved most differently at the two temperatures had the 2-AP substitutions at +1, +2, and +3. It was previously observed, that for T's in this region to acquire RNAP-dependent $KMnO_4$ sensitivity, the presence of Mg^{2+} was required (*29*). We have repeated the fluorescence experiments in buffer that not only lacked added Mg^{2+} but also contained 3 mM EDTA. The results (not shown) were similar to those presented in Figure 4, indicating that the process observed by fluorescence was not affected by Mg^{2+} ions.

The experiments whose results are displayed in Figures 2a,b, and tabulated in Table 1, were performed with the promoter constructs shown in Figure 1, which contain nine 2-AP substitutions. The amplitude of the signal obtained with these is 2−3-fold greater than that for the constructs containing only two or three 2-AP substitutions. The additional signal is important in order to be able to work at low promoter concentrations so that even at low [RNAP] it can be in sufficient excess for pseudo-first-order conditions to apply. As shown in Figure 2a, the values of k_{obs} obtained with the nine 2-AP-containing constructs at 25 °C are similar to those observed for constructs containing just two or three 2-AP in various positions. The comparison is less clear-cut for the data obtained at 10 °C (see Figure 2b), but taking into account experimental error, the rates observed for the promoter with nine 2-AP substitutions are close to those observed with the two constructs containing the more upstream 2-AP substitutions. This further supports the use of promoters containing nine 2-AP substitutions (*13*) for determining values of k_{obs} (as in Figures 2a,b and in Table 1).

Our experiments, in which clearance rates were measured, show a small but reproducible delay of escape from promoters with a functional UP element as compared to the analogous promoter without such an element. Apparently some contacts between RNAP and the UP element persist through the process of promoter clearance. However, alternative explanations such as the adoption of an UP element-dependent DNA structure that hinders promoter clearance cannot be ruled out. The rates of clearance we observed were similar to those obtained with other promoters (*30*, *31*). However, in all cases they were significantly slower than the rate of clearance at the rrnB P1 promoter in vivo, which has been estimated to initiate (and clear) with a frequency of about 1 per second. The much slower in vitro rates may reflect the participation of additional cellular proteins in the process, or solution conditions that do not adequately mimic the cellular conditions; alternatively, the nonconsensus features of rrnB P1 may be important in facilitating the rapid escape of RNAP from this promoter.

NOTE ADDED IN PROOF

Recent results indicate that at the rrnB P1 promoter, the UP element stimulates open complex formation by facilitating both RNAP binding and step(s) subsequent.

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