The effects of upstream DNA on open complex formation by *Escherichia coli* RNA polymerase

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Binding of activators to upstream DNA sequences regulates transcription initiation by affecting the stability of the initial RNA polymerase (RNAP)–promoter complex and/or the rate of subsequent conformational changes required to form the open complex (RP_O). Here we observe that the presence of nonspecific upstream DNA profoundly affects an early step in formation of the transcription bubble. Kinetic studies with the λ P_R promoter and *Escherichia coli* RNAP reveal that the presence of DNA upstream of base pair –47 greatly increases the rate of forming RP_O, without significantly affecting its rate of dissociation. We find that this increase is largely due to an acceleration of the rate-limiting step (isomerization) in RP_O formation, a step that occurs after polymerase binds. Footprinting experiments reveal striking structural differences downstream of the transcription start site (+1) in the first kinetically significant intermediate when upstream DNA is present. On the template strand, the DNase I downstream boundary of this early intermediate is +20 when upstream DNA is present but is shortened by approximately two helical turns when upstream DNA beyond –47 is removed. KMnO₄ footprinting reveals an identical initiation bubble (–11 to +2), but unusual reactivity of template strand upstream cytosines (–12, –14, and –15) on the truncated promoter. Based on this work, we propose that early wrapping interactions between upstream DNA and the polymerase exterior strongly affect the events that control entry and subsequent unwinding of the DNA start site in the jaws of polymerase.

Early steps in transcription initiation require interactions with DNA sequences located upstream from the start site (+1). In eukaryotes, assembly of the RNA polymerase (RNAP) II preinitiation complex hinges on the recruitment of the TATA-binding protein to sequences typically located ≈ 25 bp upstream from +1 (see, for example, ref. 1). In *Escherichia coli*, the specificity factor σ^{70} brings the polymerase machinery to a promoter via interactions with the so-called –35/–10 elements (2, 3). The rates and equilibria of steps involved in binding and opening the DNA are further modulated by proteins (activators, enhancers, and repressors) that bind to sites located even further upstream (1, 4). Strikingly, high-resolution structures of bacterial RNAP and yeast RNAP II reveal a conserved architecture that dictates that upstream interactions cannot place downstream DNA directly in the cleft containing the active site (5–7). Instead, significant DNA deformations must occur to access the cleft. Additionally, the width of the cleft fundamentally determines whether single- or double-stranded DNA can enter. How do interactions with upstream DNA sequences influence these critical steps or other conformational changes that occur in forming the open complex?

A paradigmatic example of the role of upstream DNA sequences in regulating transcription is found in the lysis/lysogeny decision of λ phage after infection of *E. coli* (8). DNA sequences separating two divergent phage promoters (λ P_R and λ P_{RM}) contain three sites for the phage protein λ cI. In a precise balance between λ cI concentration, site affinity, and position, transcription is repressed from λ P_R and activated at λ P_{RM} (9–11). λ cI activates λ P_{RM} by facilitating the rate-limiting conformational change (isomerization) in open complex formation, a step that occurs after polymerase binds (12–14). λ cI activation of λ P_{RM}

has been hypothesized to depend critically on the formation of a correctly aligned λ cI– σ interface (15, 16), recently defined by x-ray crystallography (16). How does formation of such an interface increase the rate-limiting isomerization rate constant (k_2) by ≈ 10 -fold at 37°C, with no net effect on the stability of the preceding intermediate (12, 14)? Additional puzzles are presented by activation of a polymerase containing a mutant σ^{70} by WT λ cI, which increases the stability of the first intermediate by ≈ 10 -fold, with only a 2-fold increase in k_2 (13). Intriguingly, modeling indicates that the transition from a nonproductive to a productive interface may alter the path of the DNA upstream of λ cI (approximately –50) (16).

These data, along with other studies, suggest to us that the trajectory of upstream DNA is defined in the earliest steps of open complex formation, and that this trajectory and the resulting interactions between upstream DNA and polymerase dictate subsequent downstream events. For *E. coli* RNAP, interactions between σ^{70} and the –35 element likely set the initial trajectory; binding to the –35 consensus sequence by the homologous σ from *Thermus aquaticus* changes the DNA helical axis by 36 degrees (17). Changes to or reinforcements of this initial trajectory presumably occur when the C-terminal domains (CTD) of the α subunits (18) and/or proteins such as catabolite activator protein (CAP), λ cI, IHF, or FIS bind upstream, bend their DNA sites (16, 19–21), and as a consequence activate transcription initiation. Possibly more significantly, the phasing of these activator sites with respect to the promoter critically affects their function (22–24). For example, repositioning of CAP at the *gal* P1 promoter by 1 bp, from a site centered at –41.5 to –40.5, converts CAP from an activator to a repressor (23). Do these experiments indicate that upstream DNA plays a direct role in activation, possibly by contacting core RNAP, or does it serve primarily as an assembly platform that mediates protein–protein interactions?

Several lines of evidence suggest that upstream DNA wraps around *E. coli* RNAP during initiation, even in the absence of transcription factors. In particular, hydroxyl radical footprinting studies of the open complex (RP_O) formed at several promoters (λ P_R, *lac*UV5, and T7 A1) reveal a pattern of periodic protection of upstream DNA extending to approximately –70 (25–27). To reconcile the length and periodicity of the RP_O footprint with the dimensions of *E. coli* RNAP determined by electron microscopy (28), upstream DNA was proposed to wrap in a surface groove of RNAP (25, 28). A similar model of RP_O at λ P_R was deduced from atomic force microscopy data (29). However, for RP_O formed at *lac*UV5, only the flexibly tethered α CTDs were found to crosslink to DNA positions –43 to –93 (30). If this crosslinking pattern is general, and α CTD binding explains upstream protection in RP_O (18, 25–27, 31–34), then it is necessary to propose rapidly reequilibrating nonspecific binding of the

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Abbreviations: RNAP, RNA polymerase; RP_O, open complex; UT, upstream truncated; FL, full length; CR, competitor resistant; t, template; nt, nontemplate; CTD, C-terminal domain; I, kinetically significant intermediate.

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α CTDs to the minor groove of DNA throughout this region (30). Consistent with, but by no means a proof of, this hypothesis, deletion or mutation of the α CTDs eliminates protection from -40 to -70 in RP_O (18, 27, 35, 36).

Although it is evident that interactions with upstream DNA influence formation of RP_O , little quantitative data exist regarding upstream contacts in the intermediates that precede it. How do these interactions influence the individual steps that form and then convert the initial polymerase–DNA complexes into RP_O ? To elucidate the role of upstream DNA in open complex formation, we compared a λP_R promoter truncated at position -47 [upstream truncated (UT)] with full-length (FL) λP_R using kinetic and footprinting experiments.

The kinetics of formation and dissociation of RP_O complexes at the λP_R promoter require a minimal three-step mechanism:

where R is free polymerase; P is promoter DNA; I_1 and I_2 are the first and second kinetically significant intermediates, respectively; and RP_O is the final open complex (37–41). (K_1 and K_3 are equilibrium constants for the first and third steps, and k_2 and k_{-2} are the forward and reverse rate constants for the rate-determining interconversions of I_1 and I_2). Our studies demonstrate that DNA sequences upstream of -47 do not greatly affect the stability of the first intermediate or the kinetics of dissociation of RP_O at λP_R . Instead, we find that they greatly accelerate the conversion of the first to the second intermediate by a factor of 35- to 60-fold. DNase I footprints reveal that the presence of upstream DNA permits downstream DNA to fully enter the active-site cleft of polymerase early in the process. These results motivate our proposal that contacts to upstream DNA sequences directly influence movements in the transcriptional machinery that place the start site in the jaws of polymerase.

Experimental Procedures

Buffers. RNAP storage buffer (25), binding buffer (Table 1) (41), wash buffer (41), 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3 (TBE buffer) (25), urea loading buffer (25), and KMnO_4 stop buffer (40) have been described. DNase I stop buffer is 200 mM EDTA and 2 M NaCl.

RNAP. *E. coli* K12 RNAP holoenzyme ($E\sigma^{70}$) was purified by modification of the Burgess and Jendrisak procedure (42, 43) and stored at -70°C in storage buffer; working stocks were stored at -20°C . Promoter-binding activities of the RNAP preparations were determined as described (41) and ranged from 36% to 77%. Binding activities were determined in parallel with the experiments described here and did not change significantly over the course of 1 month. All RNAP concentrations reported refer to the active RNAP.

λ _R Promoter DNA. The WT λ _R promoter sequence from −60 to +20, centered in a FL 190-bp fragment (−110 to +80), was isolated from plasmid pBR81 and labeled on the nontemplate (nt) strand as described (25). A 191-bp FL λ _R fragment (−102 to +89) was isolated from a pBR80 derivative by using *Xba*I and *Sma*I, and the upstream end of the template (t) strand was labeled with ³²P-dCTP by using Sequenase DNA polymerase. The 5′ overhang was filled in with dNTPs. FL λ _R used in association assays performed at 37°C was an 898-bp fragment, as described (41). We observed no difference in the kinetics of association with the 898- and 187-bp fragments (data not shown).

A UT promoter, consisting of the WT λP_R sequence from -42 to $+20$, centered in a 136-bp fragment (-47 to $+89$), was isolated from plasmid pRLG936 (31) by cleavage with *EcoRI* and *SmaI*. The upstream end of the t strand was labeled with ^{32}P -dATP. To label the downstream end of the nt strand, an UT λP_R promoter fragment (-47 to $+64$) was generated by cutting pRLG936 with *EcoRI* and filling in the 5' overhang with dNTPs. The fragment was then cut with *XhoI*, and the 3' end of the nt strand was labeled with ^{32}P -dTTP. All λP_R fragments were isolated and purified as described (25).

To label the nt strand of UT λ P_R for KMnO₄ probing, pRLG936 was cut with *Eco*RI. The resulting 5' γ phosphate was removed and replaced with γ^{32} phosphate as described (25). Proteins were phenol-extracted, and pRLG936 was cut with *Sma*I. The λ P_R fragment was precipitated, and the upstream overhang was filled in with dNTPs. UT λ P_R was isolated and purified as described in ref. 25.

Association Kinetics. Nitrocellulose filter-binding assays were performed in binding buffer by manual mixing (41). RNAP concentrations ranged from 2 to 112 nM; DNA concentrations ranged from 0.2 to 0.5 nM. To test that heparin (50 $\mu\text{g}/\text{ml}$ final) was a nonperturbing competitor for free RNAP, representative experiments were repeated by using 100 and 250 $\mu\text{g}/\text{ml}$ heparin. Equilibrium occupancy of competitor-resistant (CR) complexes and values of k_{obs} exhibited no dependence on heparin concentration, indicating that it is an inert competitor (data not shown).

Dissociation Kinetics. RNAP and DNA were mixed in binding buffer and incubated at 17 or 37°C until binding equilibrium was reached. At 37°C, 12 nM RNAP was used for FL and UT λ P_R; at 17°C, 70 nM RNAP was used with UT λ P_R and 12 nM RNAP with FL λ P_R. Irreversible dissociation was initiated by the addition of heparin (50 μ g/ml final), and 100 μ l of the reaction was removed and filtered at recorded time intervals (41). Heparin is not a perturbing competitor for the dissociation of CR complexes at FL (44) and UT λ P_R (data not shown).

Data Analysis. Observed first-order rate constants (β_{CR}) for reversible and irreversible association to form CR complexes (RP_{O} , I_2) were determined as described (41). The irreversible association rate constant (α_{CR}) was obtained from β_{CR} and the equilibrium fraction of promoter DNA in the form of CR complexes ($\theta_{\text{eq}}^{\text{CR}}$): $\alpha_{\text{CR}} = \beta_{\text{CR}} \theta_{\text{eq}}^{\text{CR}}$, where $\theta_{\text{eq}}^{\text{CR}}$ was calculated as described (41). The dependence of α_{CR} on RNAP concentration ($[\text{R}]_{\text{T}}$) is hyperbolic and $\alpha_{\text{CR}} = k_2 K_1 [\text{R}]_{\text{T}} / (1 + K_1 [\text{R}]_{\text{T}})$ [(41); see Mechanism 1]. The product $k_2 K_1$ is the composite second-order association rate constant (k_a). All fits were weighted by $1/\sigma^2$, where σ^2 is the variance of α_{CR} . $t_{1/2}$ for isomerization is given by $t_{1/2} = 0.693/k_2$. First-order dissociation rate constants (k_d), where $k_d = k_{-2}/(1 + K_3)$ (41), were determined by plotting cpm_{corr} [counts per minute at a given time (t) corrected for background retention] as a function of time. Dissociation reactions were fit to a first-order decay equation: $\text{cpm}_{\text{corr}} = (\text{cpm}_0) e^{-k_d t}$, where cpm_0 is the initial ($t = 0$) counts per minute. All kinetic data were analyzed by using SIGMAPLOT 2000 (Systat, Point Richmond, CA).

DNase I Footprinting. RNAP (70 nM) and promoter DNA (0.4–1 nM) were mixed and incubated at 17°C. To characterize I₁, samples were incubated for 15 (FL) or 350 s (UT); RP_O was characterized at 2,500 (FL) or 10,000 s (UT). At the appropriate time, heparin (50 µg/ml final) or H₂O was added, and DNase I (0.3 µg/ml final) was added 10 s later; DNase I cleavage was stopped after 10 s. Samples were prepared and loaded on sequencing gels as described in ref. 25. All DNase I footprints were reproduced in three to seven independent experiments.

Table 1. Kinetic quantities for RNA polymerase interactions with FL and UT λP_R

T, °C	λP_R	$k_a, M^{-1} s^{-1}$	k_2, s^{-1}	K_1, M^{-1}	k_d, s^{-1}
17	UT	$(9.4 \pm 1.2) \times 10^3$	$(2.3 \pm 0.1) \times 10^{-4}$	$(4.0 \pm 0.1) \times 10^7$	$(6.7 \pm 0.6) \times 10^{-5}$
	FL	$(3.4 \pm 0.2) \times 10^5$	$(1.4 \pm 0.2) \times 10^{-2}$	$(2.4 \pm 0.1) \times 10^7$	$(8.1 \pm 0.4) \times 10^{-5}$
37	UT	$(5.2 \pm 0.4) \times 10^5$	$(2.0 \pm 0.3) \times 10^{-2}$	$(2.6 \pm 0.2) \times 10^7$	$(2.0 \pm 0.7) \times 10^{-5}$
	FL*	$(3.8 \pm 0.7) \times 10^6$	$(6.6 \pm 2.7) \times 10^{-1}$	$(5.8 \pm 2.6) \times 10^6$	$(1.0 \pm 0.9) \times 10^{-5}$

Values obtained by nonlinear analysis of the dependence of α_{CR} on $[RNAP]_T$ in binding buffer (40 mM Tris, pH 8.0/10 mM $MgCl_2$ /120 mM KCl/1 mM DTT/100 $\mu g/ml$ BSA/3.125% glycerol).

*Values from ref. 41 using an 898-bp fragment; k_d value obtained with the 189-bp fragment described here.

as supporting information on the PNAS web site). Both the sensitivity to heparin and the downstream boundaries of the footprint agree with previous characterizations of a I_1^{FL} at equilibrium at 0°C (40). In sharp contrast, the downstream DNase I boundary of I_1^{UT} extends only to +2 (t) and +7 (nt), with an isolated site of protection at +27 (nt) (Fig. 2 C and D, respectively; Figs. 8 and 9, which are published as supporting information on the PNAS web site). In the footprints of I_1^{FL} and I_1^{UT} , any protection observed after the 10 s heparin challenge is attributed to the small fraction of CR complexes (see *Experimental Procedures*) present in these experiments. No end-bound complexes were detected in these footprints. Because the structures of the first kinetic intermediates differ, we continue to designate them I_1^{UT} and I_1^{FL} .

On the t strand, protection of DNA in I_1^{UT} is continuous to position -8; partial protection is observed from positions -1 to +2. Despite the lack of strong DNase I cleavage sites between positions -7 and -2 on the free DNA, comparison of weak signals with and without RNAP from at least three independent experiments suggests that this region is not protected by RNAP. Enhancements at positions -15 (t) and -16 (t) of the UT promoter are reproducibly observed; these positions are protected in the footprint of I_1^{FL} . On the nt strand, protection of I_1^{UT} is continuous to -4, with partial protection from +1 to +7, followed by cleavage of the backbone from +8 to +26. The DNA backbone at +27 is reproducibly protected in all footprints of I_1^{UT} . Gels run for short times to resolve bands downstream from

+27 demonstrate that this isolated site of protection is not the result of an end-bound RNAP (data not shown).

In the upstream direction, DNase I protection of I_1^{UT} extends at least to -45 (nt), essentially the end of the fragment. The protection of I_1^{FL} extends to at least -52 (t) and -45 (nt). In I_1^{UT} , DNase I enhancements are located at positions -23 (nt) and -34 (nt); in I_1^{FL} , enhancements occur at -22 and -45 (nt). Fractional protection of the upstream (-35 to -3) and downstream ($+2$ to $+27$) regions of the I_1^{FL} footprint is the same within error (see Figs. 6 and 7). Thus under these conditions, as well as at equilibrium at 0°C (40), DNase I footprinting detects a homogeneous population of competitor-sensitive complexes with extended downstream footprints on FL λP_R .

At binding equilibrium, RP_O formed on FL and UT λP_R (Figs. 6–9 and Fig. 10, which is published as supporting information on the PNAS web site) have the same downstream boundaries: +20 (t) and $\sim +25$ (nt). As reported (25), DNase I enhancements occur at -38 (t), -48 (t), and -45 (nt) in RP_O formed on the FL promoter, indicative of DNA deformations (e.g., bending); no enhancements are observed at any position in RP_O formed on the UT promoter. No end-bound complexes were detected on UT or FL λP_R .

Differences in UT and FL RP₀ as Detected by KMnO₄. Equilibrium complexes formed at 37°C and 17°C were next probed with KMnO₄ to estimate the span of the initiation bubble and the pyrimidines (T ≫ C) that are solvent-accessible in RP₀. For both FL and UT

Fig. 2. Differences in the downstream boundary of I_1^{FL} versus I_1^{UT} . Representative scans of DNase I cleavage of free DNA and of I_1^{FL} and I_1^{UT} complexes ($\pm$ heparin challenge) formed at 17°C on FL [t strand (A); nt strand (B)] or UT λP_R [t strand (C); nt strand (D)]. I_1^{FL} was probed at 15 s; I_1^{UT} was probed at 350 s.

DNA backbone in this region is also distorted in I_1^{UT} . Although the $KMnO_4$ reactivity detected at these positions could result from a small population of off-pathway complexes formed on $UT \Delta P_R$, the lack of a similar shift in $KMnO_4$ reactivity of the nt strand suggests that this is unlikely.

A model of I_1^{FL} proposes that formation of a sharp kink at $-11/-12$ directs downstream DNA into the jaws created by the β and β' subunits of RNAP (41), thus protecting it from DNase I cleavage to $\approx +25$ (40). The observation that the DNase I footprint of I_1 on the UT promoter is shorter by two helical turns indicates that either the DNA is not properly placed in the jaws and/or the jaws are not in the appropriate conformation to make extensive downstream contacts. Complexes formed at low temperature at other promoters, thought to correspond to early competitor-sensitive intermediates (referred to as RP_C), exhibit even shorter downstream DNase I footprints (to ≈ -5) (7, 23, 26, 54). Darst and coworkers (7) inferred from these data and their structure of the bacterial RNAP bound to a 33-bp forked-junction promoter fragment (from -40 to -7) that DNA downstream of the -10 region in RP_C is not yet bent into the jaws but instead passes above them. A superposition of the DNA backbone bound in the active site of DNase I [Protein Data Bank ID code file 1DNK (55)] onto the DNA bound in RP_C (7) demonstrates that any position downstream of -5 can be cleaved. To explain protection extending to $+2$ (t)/ $+7$ (nt) in I_1^{UT} , we hypothesize that downstream DNA must be bent into the jaws, but that its disposition fundamentally differs from that in

I_1^{FL} . The isolated site of protection at $+27$ (nt) in I_1^{UT} may also be consistent with an altered downstream DNA trajectory or change in the position of the downstream jaw (see below).

Interestingly, the downstream DNase I boundary of I_1^{UT} is very similar to that of the unusual open complex formed at the T7 A2 promoter DNA by a variant *E. coli* RNAP [$+1$ (t) and $+8$ (nt); (56)]. This variant contains a large deletion in β ($\Delta 186-433$) that essentially removes the downstream lobe of the jaw. The downstream footprint of I_1^{UT} may also reflect an altered conformation of the downstream jaw resulting from the loss of upstream contacts. In free holoenzyme, the negatively charged N-terminal domain of σ^{70} (region 1.1) has been proposed to bind in this region of the jaws (57) and thereby prop them open (58, 59). Possibly the absence of upstream contacts somehow impedes the expulsion of region 1.1 from the active site cleft, a transition required to form RP_O . In this scenario, which is currently being investigated, downstream DNA would be bent into the jaws in I_1^{UT} , but prevented from descending into them by σ^{70} .

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