Sequence-independent upstream DNA–αCTD interactions strongly stimulate *Escherichia coli* RNA polymerase-*lacUV5* promoter association

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The C-terminal domains of the two α-subunits (αCTD) in *Escherichia coli* RNA polymerase (RNAP) recognize specific sequences called UP elements in some promoters. These interactions can increase transcription dramatically. Previously, effects of upstream DNA–αCTD interactions on transcription were quantified relative to control promoters with nonspecific DNA sequences substituted for UP elements. However, contributions of nonspecific upstream DNA–αCTD interactions to promoter activity have not been evaluated extensively. Here, we examine effects of removal of αCTD, upstream promoter DNA, or both on the rate of open-complex formation with promoters that lack UP elements. Deletion of αCTD decreased the composite second-order association rate constant, k_a, of RNAP for the *lacUV5* promoter by ≈10-fold. Much of this effect was attributable to a decrease in the isomerization rate constant, k_2. Removal of promoter DNA upstream of the −35 element also decreased both k_a and k_2 ≈10-fold. Upstream DNA extending approximately to base pair −100 was sufficient for maximal association rates of wild-type RNAP with *lacUV5* promoter fragments. The αCTD and upstream DNA did not affect dissociation rates from the open complex. We suggest that sequence-independent upstream DNA interactions with αCTD are major contributors to initiation at many (or all) promoters (not merely promoters containing UP elements) and that these interactions facilitate isomerization events occurring well downstream of the α-binding sites. In addition to highlighting the functional importance of nonspecific protein–DNA interactions, these results suggest also that UP element–αCTD interactions play an even larger role in transcription initiation than appreciated previously.

α-subunit | nonspecific interactions | transcription initiation | isomerization

RNA polymerase (RNAP) interacts with 70–80 bp of DNA in open complexes formed at *Escherichia coli* promoters, spanning a region from ≈50–60 bp upstream to 20 bp downstream of the transcription-initiation site (1–2). Each of the four principal RNAP subunits (σ, α, β, and β′) contacts promoter DNA. The specificity subunit (σ) contacts at least three promoter regions: the −10 hexamer, extended −10 region, and −35 hexamer (1). The β- and β′-subunits form the catalytic center of the enzyme and contact DNA in the vicinity of and downstream from the transcription-start site (3–5).

All previously characterized contacts of RNAP with "upstream DNA" (defined as DNA located upstream of the −35 hexamer) are mediated by the C-terminal domains of the two α-subunits (5–7). The αCTDs bind in a sequence-specific manner at two preferred positions in the A+T-rich upstream DNA sequences referred to as UP elements, the "proximal" (centered at approximately −41) and "distal" (centered at approximately −52) UP-element subsites (8). RNAP lacking the αCTD can be reconstituted *in vitro* and retains the ability to recognize promoters and transcribe RNA, but it is defective in responding to UP elements and many activators (6, 9, 10).

UP elements have been characterized in several bacterial species and can increase promoter activity dramatically (>50-fold *in vivo*

relative to the same core promoter with other upstream sequences) (6, 8, 11–16). An UP-element consensus sequence (Fig. 1) that increased transcription >100-fold was identified by using *in vitro* selection, followed by a screen for function *in vivo* (8, 10, 15).

The ≈8-kDa αCTD contains two helix–hairpin–helix motifs that bind to the DNA minor groove (17–20). Genetic studies (18) and interference and protection footprints (21) are in agreement with a high-resolution αCTD–DNA x-ray structure (19) concerning the mechanism of αCTD–DNA recognition. DNA recognition involves a number of residues in αCTD, most notably R265, whose side chain contacts the DNA backbone and makes water-mediated interactions with bases in the minor groove. The orientation of an αCTD with respect to the core promoter can vary and can be influenced by stabilizing interactions with σ region 4.2 and/or DNA-bound transcription factors (22–25).

The general mechanism of RNAP binding to a promoter consists of initial binding to form a closed complex, followed by conformational changes to form one or more additional intermediates before formation of the open complex (RP_O) in which the DNA strands are unpaired to expose the transcription-initiation site (1, 26–28). The UP element in the rRNA promoter *rrnB* P1 increases the composite second-order association-rate constant, k_a, ≈30-fold relative to the same core promoter with other DNA sequences substituted for the UP element (12). Most of this increase results from stimulation of initial closed-complex formation, although the *rrnB* P1 UP element might also affect isomerization (12). The *rrnB* P1 UP element also has an ≈10-fold effect on the isomerization rate constant of the λP_{RM} promoter in a chimeric construct (29–30).

In addition to interacting specifically with UP elements, αCTD also interacts nonspecifically with upstream DNA in promoters that lack UP elements. These include the well characterized *lacUV5* and λP_R promoters, in which upstream sequences do not closely match the UP-element consensus (Fig. 1) and do not function in a sequence-specific manner (31). Replacement of these upstream sequences with other non-UP-element sequences had little (<2-fold) if any effect on promoter activities *in vitro* or *in vivo* (12, 29, 31). Nevertheless, αCTD-dependent interactions with upstream DNA were identified in RNAP footprints of *lacUV5* and λP_R (7, 31) and in crosslinking experiments with *lacUV5* (5). At two other promoters that lack UP elements, *galP1* and λP_{RM}, reduced levels of open (KMnO₄-reactive) complexes were observed with RNAP lacking αCTD relative to wild-type RNAP (29, 32).

Here, we report a kinetic analysis of the role of RNAP interactions with nonspecific upstream promoter DNA *in vitro*, using wild-type and αCTD-mutant RNAPs and promoter fragments of varying length. Our results suggest that upstream DNA interactions with αCTD play a role in the rate of RNAP
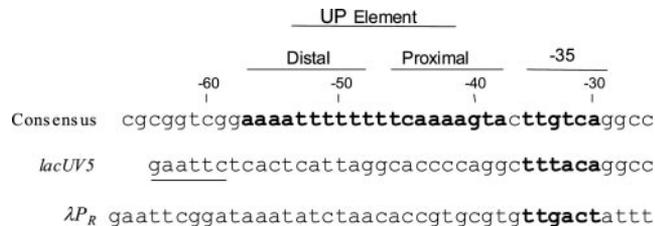
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Fig. 1. Sequences upstream of the −35 element in the *lacUV5* and λ*P_R* promoters, compared with the *in vitro*-selected full-consensus UP element (8). The −35 hexamers and consensus UP element are in shown in bold. Positions of the proximal and distal UP-element subsites are indicated. *Eco*RI sites used in cloning the promoters into plasmid vectors are underlined. Sequences upstream of −60 in the promoter fragments were obtained from plasmids pRLG770 or pSL6 (34–35) and are shown in Fig. 7. Numbering is with respect to the transcription start site.

association at all promoters, both with and without UP elements. Furthermore, because the quantitative effects of UP elements on association of RNAP with promoters were determined previously relative to effects of nonspecific DNA (rather than to effects of removal of αCTD), our results suggest that UP element–αCTD interactions can have an even larger effect on transcription initiation than appreciated previously.

Materials and Methods

RNAP Preparations. Wild-type native RNAP holoenzyme, purified by standard procedures (33), was a generous gift from R. Landick (University of Wisconsin, Madison). Wild-type, αΔ235, and αR265A RNAPs containing N-terminally histidine-tagged α-subunits were reconstituted *in vitro* from overexpressed subunits as described (18, 21). Active RNAP concentrations were determined by promoter fragment titration (26) as follows: 2 nM ^{32}P-end-labeled *lacUV5* promoter fragment (−100 to +40 or −130 to +40) was incubated with RNAP (1–20 nM total protein) at 37°C for 40 min in 10 mM Tris·Cl, pH 8.0/30 mM KCl/10 mM MgCl$_2$/1 mM DTT/100 μg/ml BSA. Heparin (Sigma) was added to 10 μg/ml, and after 30 s, reactions were filtered. Filters (0.45 μm; NC45, Schleicher & Schuell) were then washed, dried, and quantified by Cerenkov counting. Wild-type native RNAP was ≈70% active in this assay, and reconstituted RNAPs were 20–30% active. Three reconstituted preparations of each RNAP were tested, and similar results were obtained. All calculations are based on active concentrations of RNAP, and comparisons are always between wild-type and mutant RNAPs purified by the same method.

Promoter Fragments. Fragments were prepared from restriction digests of pRLG4264 or DNA amplified from pRLG593 by PCR using vector-specific primers (21). pRLG4264 contains *lacUV5* sequence from −59 to +38, inserted as an *Eco*RI-*Hin*dIII fragment into pSL6 (34). pRLG593 contains the same *lacUV5* fragment inserted into pRLG770 (6, 35). Vector sequences upstream of −59 are shown in Fig. 7, which is published as supporting information on the PNAS web site. *lacUV5* fragments were 3′-end-labeled in the nontemplate strand at the *Hin*dIII site (+40) with Sequenase (United States Biochemical); [α-^{32}P]dATP; and unlabeled dGTP, dCTP, and TTP to produce a blunt end. Upstream end points were derived from the PCR primer for the −100 to +40 fragment used in Fig. 8 (which is published as supporting information on the PNAS web site) or from the following restriction digests of pRLG4264 (for fragments shown in Figs. 2 and 4–6): −130, *Eco*RV; −100, *Ssp*I; −63, *Eco*RI; −45, *Nla*IV; and −42, *Bsa*JI (Figs. 1 and 7). The 5′ overhanging ends were filled in with unlabeled dNTPs and Sequenase to produce blunt ends. The λ*P_R* fragments (−110 to +80; λ*P_R* sequences from −60 to +20 embedded in vector sequence) were
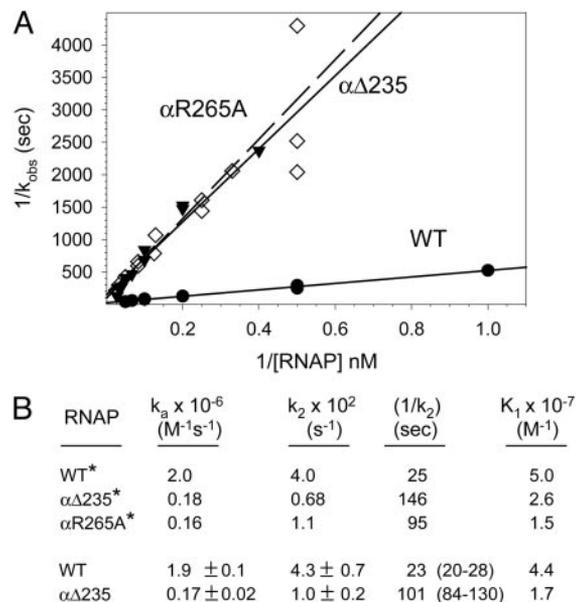
prepared from *Bss*HII–*Sma*I digests of pBR81 and 3′-end-labeled at the *Bss*HII site with [α-^{32}P]dCTP and Sequenase (36, 37). Fragments were purified from acrylamide gels (21).

Association Kinetics. Rates of formation of heparin-resistant complexes were determined by using a filter binding assay (26, 28). RNAP (always in ≥3-fold molar excess over promoter DNA) and promoter fragment (<1 nM) were incubated at 25°C (Figs. 3 and 8) or 30°C (all other figures) in the buffer described above, except containing either 40 mM KCl (Fig. 8) or 110 mM KCl (all other figures). Aliquots were removed at time intervals to tubes containing heparin (final 10 μg/ml), filtered, and counted as described above. Counts were corrected for background retained in the absence of RNAP. The fraction of heparin-resistant complexes was determined as a fraction of input counts and plotted vs. time. Observed first-order rate constants, k_{obs}, were determined from fitting to a single exponential [cpm$_{obs}$ = (cpm$_{plateau}$)($1 - e^{-k_{obs}t}$)], where cpm$_{plateau}$ is the fraction of counts retained at equilibrium (23, 28). Maximum plateau values were ≈65% for *lacUV5* and 80% for λ*P_R*. Under conditions of reversible association, observed rate constants were corrected for the equilibrium fraction of heparin-resistant complexes (28).

Data were interpreted by using the previously characterized mechanism for RNAP association with the *lacUV5* promoter under these conditions (27), and they are shown as τ plots ($1/k_{obs}$ vs. $1/[RNAP]$), where RNAP refers to total active RNAP (Figs. 2–5 and 8), or as nonlinear plots (k_{obs} vs. [RNAP]; Figs. 9 and 10, which are published as supporting information on the PNAS web site). k_a (composite second-order association rate contant) and k_f (first order isomerization rate constant) were determined from τ plots by using the equation $1/k_{obs} = (1/k_a[RNAP]) + 1/k_f$, or they were determined from nonlinear plots by using the equation $k_{obs} = k_a k_f [RNAP]/(k_a[RNAP] + k_f)$. K_1 ($\cong K_B$) was determined from k_a and k_f ($K_1 = k_a/k_f$). Data were fit by using SIGMAPLOT 8.0, and errors were determined from a weighted nonlinear analysis (28). The rate

Fig. 2. Effects of αΔ235 or αR265A on association of RNAP with the *lacUV5* promoter. (*A*) Double reciprocal (τ) plots (see text) from reactions at a range of RNAP concentrations with the −130 to +40 fragment at 30°C. Wild-type reconstituted RNAP (●), αΔ235 RNAP (◇), and αR265A RNAP (▼) are indicated. (*B*) Kinetic constants derived from the data shown in *A* (asterisks) or from nonlinear analysis of the same data (no asterisks; see also Fig. 9). Data from αR265A RNAP could not be fit for nonlinear analysis.

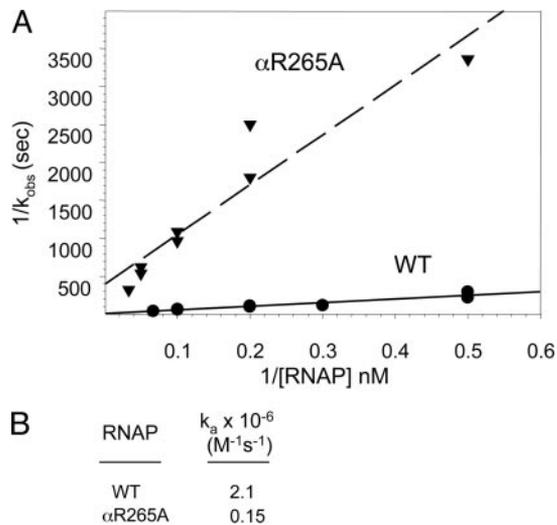| RNAP	$k_a \times 10^{-6}$ ($M^{-1}s^{-1}$)	$k_2 \times 10^2$ (s^{-1})	$(1/k_2)$ (sec)	$K_1 \times 10^{-7}$ (M^{-1})
WT*	2.0	4.0	25	5.0
αΔ235*	0.18	0.68	146	2.6
αR265A*	0.16	1.1	95	1.5
WT	1.9 ± 0.1	4.3 ± 0.7	23 (20-28)	4.4
αΔ235	0.17 ± 0.02	1.0 ± 0.2	101 (84-130)	1.7

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Fig. 3. Effects of αR265A on association of RNAP with the λP_R promoter (−110 to +80). (*A*) Shown are τ plots (see text) from reactions at 25°C. Wild-type reconstituted (●) and αR265A (▼) RNAP are indicated. (*B*) k_a was derived from the linear analysis in shown in *A*.

constant $k_f \cong k_2$ under these conditions (30°C) and reflects the rate-limiting step in the formation of RP$_O$, the isomerization of RP$_C$ to RP$_i$ (27). The nomenclature K_1 ($\cong K_B$) and k_2 ($\cong k_f$) is used here.

Dissociation Kinetics. RNAP (10 nM) was incubated with promoter fragments for 15 min at 30°C in the buffer described above (with 110 mM KCl). After heparin addition to 10 μg/ml, aliquots were removed at intervals, filtered, and counted. Half-lives of open complexes were determined from plots of ln(fraction remaining) vs. time. Dissociation rate constants, k_d, were determined from the first-order decay equation, cpm$_{retained}$ = (cpm$_{max}$)$e^{-k_d t}$. Equivalent results were obtained with heparin at 10 μg/ml or 25 μg/ml and

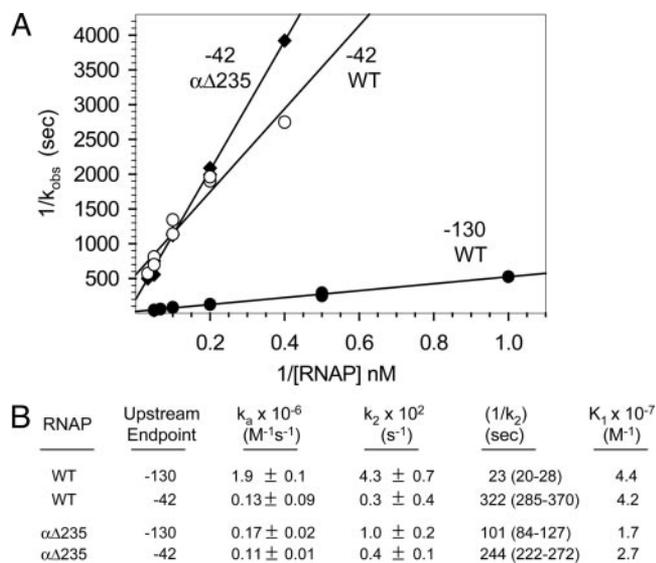**Fig. 4.** Effects of truncation of upstream DNA (to −42) on association of wild-type RNAP or αΔ235 RNAP with the *lacUV5* promoter (30°C, 110 mM KCl buffer). (*A*) Data are displayed as a τ plot. Wild-type reconstituted RNAP and −130 to +40 *lacUV5* promoter fragment (●, from Fig. 2), wild-type reconstituted RNAP and −42 to +40 *lacUV5* fragment (○), and αΔ235 RNAP and −42 to +40 *lacUV5* fragment (◆) are indicated. (*B*) Kinetic constants derived from nonlinear analysis of the data (see also Fig. 9).
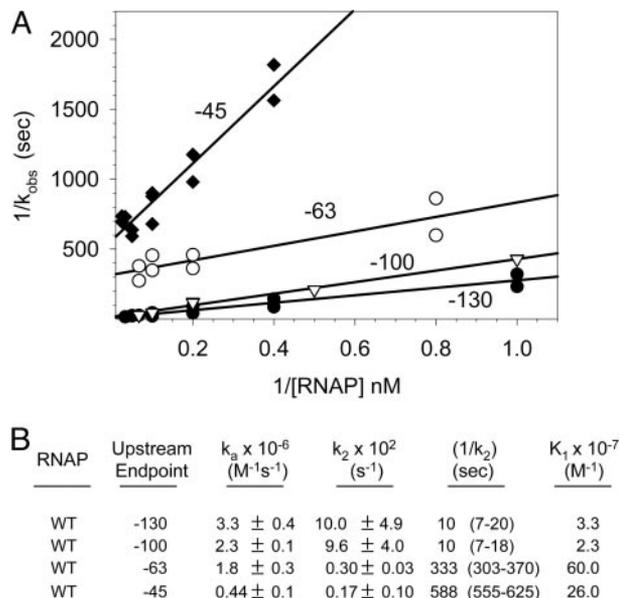

Fig. 5. Effects of upstream DNA length on association of wild-type native RNAP with the *lacUV5* promoter (30°C, 110 mM KCl buffer). (*A*) Promoter fragments were truncated at −130 (●), −100 (▽), −63 (○), or −45 (◆). The downstream end point for each promoter fragment was +40. Data are displayed as τ plots. (*B*) Kinetic constants derived by nonlinear analysis of the data (see also Fig. 10).

with 200 nM double-stranded DNA (a 60-bp "full-consensus" promoter fragment) (38) as competitor (data not shown).

Results

Deletion of αCTD Reduces the RNAP Association Rate. To determine the contribution of αCTD to open-complex formation at promoters that lack UP elements, the rates of formation of heparin-resistant complexes at the *lacUV5* promoter were determined as a function of concentration of reconstituted wild-type RNAP or RNAP lacking the αCTD (αΔ235 RNAP) by using a filter binding assay (26) (see *Materials and Methods*). We used *lacUV5* for these studies because it lacks a sequence-specific UP element (31), and its kinetics (27) and interactions with the RNAP α-subunit (5, 7) have been characterized extensively.

First-order rate constants for binding to a *lacUV5* promoter fragment (−130 to +40) were determined in RNAP excess at several concentrations of RNAP by using solution conditions in which rates could be measured for both enzymes (30°C, 110 mM KCl). The data are presented as a double-reciprocal (τ) plot (39) (Fig. 2*A*) in which the slope is the reciprocal of the composite second order rate constant k_a ($1/k_a = 1/K_1 k_2$), and the y-intercept is the reciprocal of the isomerization rate constant k_2 ($1/k_2$) (see *Materials and Methods*). This linear plot facilitates visualization of differences in the rates of formation of the complexes, but whenever possible, the kinetic constants are provided from nonlinear analysis of the data (28) (Figs. 2*B* and 9), which provides a more accurate estimate of k_2 and experimental error.

The overall association rate for αΔ235 RNAP and the *lacUV5* promoter was ≈10-fold slower than for wild-type RNAP ($k_a = 1.7 \times 10^5$ M^{-1} s^{-1} for αΔ235 RNAP vs. 1.9×10^6 M^{-1} s^{-1} for wild-type RNAP). k_2 for αΔ235 RNAP was ≈4-fold slower than for wild-type RNAP [τ-intercept values $(1/k_2) = 101$ s for αΔ235 RNAP and 23 s for wild-type RNAP]. The kinetic constants for wild-type reconstituted RNAP were similar to those for wild-type native RNAP (see below) and to those obtained previously

by investigators using different methods but similar solution conditions (27).

The overall association rate was also measured for a mutant RNAP containing a single amino acid substitution in αCTD (R265A) that eliminates DNA binding to the same degree as deletion of the entire C-terminal domain (6, 18). k_a for αR265A RNAP was very similar to that for αΔ235 RNAP (Fig. 2). This result is consistent with the conclusion that DNA binding by αCTD is required for the effect of αCTD on the association rate, although additional interactions between αCTD and some other part of RNAP cannot be excluded.

Similar conclusions were reached from experiments with a *lacUV5* fragment containing different vector-derived sequences in the upstream region, a slightly different upstream DNA end point (−100), different temperature (25°C) and salt (40 mM KCl) conditions, and different reconstituted RNAP preparations (Fig. 8). Again, the overall k_a values for αΔ235 RNAP and αR265A RNAP were 10-fold lower than for wild-type RNAP and isomerization rates were slower for mutant than for wild-type RNAP. The fold difference in k_2 cannot be determined with certainty in this experiment because $1/k_2$ observed for wild-type RNAP under these conditions is indistinguishable from zero.

Similar results were obtained also with another promoter lacking an UP element, λP_R (Fig. 3). In this case, k_a for αR265A RNAP was reduced ≈14-fold compared with wild-type RNAP. k_a for wild-type reconstituted RNAP (2.1×10^6 M^{-1} s^{-1}) was consistent with that determined previously for wild-type native RNAP and λP_R under similar conditions (7.2×10^5 M^{-1} s^{-1}) (26, 28). Although the τ plot suggests that a reduction in k_2 is the primary effect of removal of αCTD (Fig. 3), experimental uncertainty in the data does not allow a definitive conclusion. However, other results (36) strongly support the inference that αCTD binding to upstream DNA in λP_R increases k_2.

Elimination of Upstream DNA Reduces the Overall Association and Isomerization Rate Constants for *lacUV5*. If the results given above reflect loss of sequence-independent upstream DNA interactions with αCTD, then removal of upstream DNA sequences should have effects similar to elimination of αCTD. Consistent with this prediction, the rate of association of wild-type reconstituted RNAP and a *lacUV5* fragment extending only to −42 was ≈10-fold slower than for the fragment extending to −130 ($k_a = 1.3 \times 10^5$ vs. 1.9×10^6 M^{-1} s^{-1}) (Figs. 4 and 9), similar to the effect of deleting αCTD (Fig. 2). Most of the effect of removal of upstream DNA resulted from a reduction in the isomerization rate constant ($k_2 = 4.3 \times 10^{-2}$ s^{-1} vs. 0.3×10^{-2} s^{-1}), consistent with the relatively large effect of deletion of αCTD on k_2 (Fig. 2). Wild-type RNAP associated slightly faster with a fragment extending to −45 than with the −42 fragment (data not shown), consistent with the presence of an αCTD-binding site centered at −41 (5, 7).

In contrast to the large effects of removal of upstream DNA on the kinetics of association with wild-type RNAP, removal of upstream DNA had smaller effects on k_a and k_2 with αΔ235 RNAP ($k_a = 1.7 \times 10^5$ vs. 1.1×10^5 M^{-1} s^{-1}, and $k_2 = 1.0 \times 10^{-2}$ vs. 0.4×10^{-2} s^{-1} for the −130 and −42 fragments, respectively) (Fig. 4B). The kinetic constants for the wild-type and αΔ235 RNAPs on a promoter fragment lacking upstream DNA (−42 to +40) also differed very little ($k_a = 1.1 \times 10^5$ vs. 1.3×10^5 M^{-1} s^{-1}, and $k_2 = 0.3 \times 10^{-2}$ vs. 0.4×10^{-2} s^{-1}, respectively) (Figs. 4 and 9).

Upstream DNA Length Required for Stimulation of RNAP Binding to the *lacUV5* Promoter. To determine the length of upstream DNA required for maximal rates of association with RNAP, experiments were carried out by using *lacUV5* fragments with upstream end points of −45, −63, −100, or −130 and wild-type native RNAP (Fig. 5). The kinetic constants k_a, K_1, and k_2 for the two longer fragments (−100 to +40, −130 to +40) were very similar

(Fig. 5) and in good agreement with those determined previously for a 211-bp *lacUV5* fragment (−140 to +63) by using similar solution and temperature conditions and an abortive initiation assay (27).

Both k_a and k_2 for binding of RNAP to the promoter fragment extending to −45 were greatly decreased compared with the two longest fragments (Fig. 5), consistent with the effects observed with the −42 fragment shown in Fig. 4. For the intermediate-length fragment (−63), k_2 was also significantly decreased relative to the longer fragments, although the reduction in k_a was not as great as that observed with the −45 fragment as a consequence of an apparent increase in K_1 (see *Discussion*). Together, the data indicate that sequences extending to −100 are sufficient for maximal rates of association, and that sequences at, or upstream of, −63 play a large role in determining the isomerization rate k_2 (see *Discussion*).

To determine whether open complexes were formed with the short fragments, heparin-resistant complexes formed on fragments extending to −130, −63, and −45 were compared in KMnO$_4$ footprints. The same KMnO$_4$-reactive positions were observed on the template and nontemplate strands of each of the three promoter fragments (Fig. 7 and Fig. 11, which is published as supporting information on the PNAS web site); no differences in the identities or relative reactivities of signals were observed as a function of upstream length (see *Discussion*).

Dissociation Rates of *lacUV5* Complexes Are Unaffected by Upstream DNA or αCTD. Dissociation rates of heparin-resistant *lacUV5* promoter complexes were determined as a function of αCTD and upstream DNA length by using the filter binding assay. Complexes formed with wild-type native RNAP and *lacUV5* fragments extending to −45, −63, −100, or −130 were long-lived and did not differ significantly from each other (110- to 130-min half-lives). k_d values (≈1×10^{-4} s^{-1}; Fig. 6) were very similar to that reported previously for experiments that used similar solution conditions (27). Complexes formed with reconstituted αΔ235, αR265A, or wild-type RNAPs and *lacUV5* fragments extending to −130 or −42 were long-lived and had similar dissociation constants (Fig. 6 *B* and *C*, and data not shown). These results indicate that the rate-limiting step(s) in decay of the *lacUV5* open complex to a competitor-sensitive intermediate are not affected by the nonspecific upstream DNA–αCTD interactions that have such profound effects on the association kinetics.

Discussion

Significance of αCTD-Nonspecific DNA Interactions for Association of RNAP. Because the αCTD is essential for viability (18) and upstream DNA cannot be removed from a promoter *in vivo*, we used *in vitro* kinetic approaches with known concentrations of active enzyme to quantify sequence-independent effects of αCTD–upstream DNA interactions. We found that removal of αCTD, upstream DNA, or both reduced the overall k_a for RNAP with *lacUV5* ≈10-fold. A major part of this effect was on isomerization of a heparin-sensitive intermediate (RP$_c$) to a heparin-resistant complex (RP$_i$) that is in rapid equilibrium with the open complex (RP$_O$) under these conditions (27), without a concomitant decrease in the dissociation rate. Loss of αCTD–DNA interactions also resulted in a 14-fold reduction in k_a for λP_R, another promoter lacking an UP element. Together, these findings suggest that αCTD–upstream DNA interactions are likely to play a significant role in RNAP binding at many or all promoters, not merely promoters that have UP elements or that use transcription factors. This conclusion is consistent with previous observations that αCTD mutant RNAPs formed lower levels of KMnO$_4$-reactive complexes than wild-type RNAP at other promoters without UP elements (29, 32). The importance of the αCTD–DNA interaction for RNAP association at many
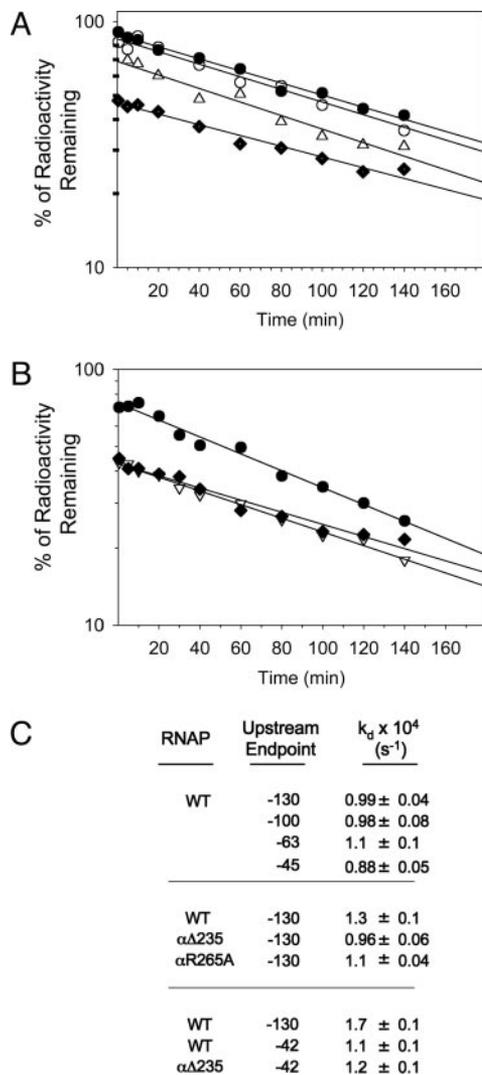
Fig. 6. Effects of extent of upstream DNA and αCTD on dissociation rates from *lacUV5*. The fraction of remaining open complexes is plotted as a function of time after addition of heparin. (*A*) Dissociation of native wild-type RNAP from *lacUV5* fragments with upstream end points −130 (●), −100 (○), −63 (▽), or −45 (◆). Downstream end points were all +40. (*B*) Dissociation of reconstituted wild-type (●), αR265A (▽), and αΔ235 (◆) RNAPs from *lacUV5* (−130 to +40). (*C*) k_d values were derived from data shown and from a *lacUV5* promoter fragment (−42 to +40) (data not shown), as described in *Materials and Methods*.

RNAP	Upstream Endpoint	$k_d \times 10^4$ (s^{-1})
WT	−130	0.99 ± 0.04
	−100	0.98 ± 0.08
	−63	1.1 ± 0.1
	−45	0.88 ± 0.05
WT	−130	1.3 ± 0.1
αΔ235	−130	0.96 ± 0.06
αR265A	−130	1.1 ± 0.04
WT	−130	1.7 ± 0.1
WT	−42	1.1 ± 0.1
αΔ235	−42	1.2 ± 0.1

or all promoters, in addition to its importance for transcription initiation and elongation factor function (10, 40), is likely to contribute to the requirement for αCTD for cell viability (18).

DNA-binding proteins exhibit sequence-nonspecific as well as sequence-specific interactions (e.g., *lac* repressor) (41–42), and although nonspecific interactions are generally electrostatic and weaker, they can be crucial for regulation *in vitro* and *in vivo*. Nonspecific binding can influence the effective concentration of a protein and facilitate binding-site location by sliding, intersegment transfer, and/or hopping (43). Because the αCTDs are covalently tethered to RNAP, the locations of specific binding sites in promoters with UP elements and of nonspecific binding sites in promoters like *lacUV5* are essentially the same (as indicated by hydroxyl radical footprints) (7, 8). The affinity of α for UP element DNA was estimated to be at least 15-fold greater than for nonspecific DNA (18), and we suggest that the larger effects of UP elements than nonspecific αCTD-binding sites reflect larger increases in K_1, as observed at *rrnB* P1 (12).

Implications for UP-Element Function. The magnitude of UP-element effects on transcription or on RNAP association rates with promoters was determined previously by comparing the effects of UP element sequences to those of nonspecific sequences in the context of the same core promoter (for e.g., see refs. 6, 12, 15, 16, and 31). The results presented here suggest that because nonspecific interactions with αCTD increase the rate of RNAP association, it is likely that the previously determined effects of UP elements underestimated the overall contribution of αCTD–UP element interactions to promoter function: the specific interaction amplifies the effect of the nonspecific interaction. For example, as much as a 1,000-fold increase in k_a would be predicted for the consensus UP-element sequence fused to the *rrnB* P1 core promoter relative to the same promoter truncated upstream of the −35 element or transcribed by RNAP lacking αCTD.

Length of Upstream DNA Required for Effects on RNAP Association. Deletion of DNA upstream of the −35 element had a large effect on the association of RNAP with *lacUV5*, affecting both k_a and k_2. Surprisingly, most of the large and unexpected effect on k_2 derived from sequence between −63 and −100. The precise location of the critical sequence in this region has not been defined further.

The requirement for sequences upstream of the −35 hexamer reflects interactions with αCTD. Crosslinks of αCTD to upstream DNA occur at periodic intervals from approximately −41 to approximately −95 (5), and αCTD-dependent footprint protections extend upstream to at least −70 (7, 44). Hydroxyl radical footprints of RNAP complexes with the −130 *lacUV5* fragment (Fig. 12, which is published as supporting information on the PNAS web site) indicate partial occupancy of three principal sites upstream of −35 (at −41, −52, and −63), consistent with previous reports (7).

Although sites corresponding to the UP-element proximal and distal subsites (at −41 and −52) (8) were occupied on the fragment truncated at −63 (Fig. 12), the isomerization rate for this fragment was much slower than for the full-length fragment. Sequences upstream of −63 provide additional potential binding sites for αCTD. The third principal αCTD-binding site, centered at −63, is truncated on this fragment, suggesting that this particular site might be important for *lacUV5* function. Consistent with this possibility, the distal subsite of the *rrnB* P1 UP element (−52) was fully functional when displaced upstream to −63, although not when moved further upstream (45).

Although most of the effect of upstream sequences can be attributed to interactions with αCTD, we cannot exclude the possibility that DNA upstream of the αCTD sites could interact with another surface on RNAP. Crosslinks to an additional RNAP surface were not observed in *lacUV5* open complexes with wild-type RNAP (5) (although potential transient interactions with an early transcription intermediate would not have been detected in these experiments). Deletion of upstream DNA had a small effect even with αCTD-deleted RNAP (Fig. 4) (although this effect could be attributable to a DNA-binding surface not accessible on wild-type RNAP). Potential contacts between DNA several turns upstream of the −35 element and another surface on RNAP could depend on αCTD–DNA interactions. It has been suggested that transcription factors could play this role at some promoters (46, 47).

Effects of Upstream DNA on Individual Kinetic Constants. Elimination of αCTD reduced the equilibrium constant for the initial step, K_1 (≈2- to 3-fold) (Figs. 2 and 4), consistent with the stimulatory effects of UP elements on K_1 observed previously at rRNA promoters (12). However, K_1 was not reduced by deletion of upstream DNA: with reconstituted wild-type RNAP, there was no effect of DNA removal on K_1 (Fig. 4), whereas with native RNAP, there was an increase in K_1 (Fig. 5). It is possible that this difference in the

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effects on K_1 with the two wild-type RNAPs reflects the presence or absence of other factor(s) in the enzyme preparations. For example, the ω-subunit, present in native but not reconstituted RNAP, has been proposed to facilitate RNAP assembly (48) and could result in subtle differences in the RNAPs.

The mechanism that is responsible for the effect of upstream DNA deletion on K_1 with wild-type native RNAP is unclear. An increase in K_1 was observed also with native RNAP upon truncation of a λP_R promoter fragment, where it was attributed to reductions in the energetic costs of establishing contacts with core promoter DNA (36). Although less likely, effects are also possible from potential end-binding of RNAP to fragments whose upstream end points are close to the core promoter. However, we emphasize that the overall consequences of deleting upstream DNA correlate with the effects of removal of the αCTD; large reductions in k_a and k_2 occur in each case.

It is likely that the isomerization step, k_2, involves conformational changes that occur far downstream of the DNA regions directly contacted by αCTD. We have observed differences in the extent of protection by RNAP in the extended −10 region (as well as in the upstream region) when comparing $rrnB$ P1 footprints with αCTD mutant vs. wild-type RNAP (ref. 49 and W.R. and R.L.G., unpublished data). The accompanying article (36) reports differences in the extended −10 region as a function of DNA upstream of λP_R in $KMnO_4$ footprints. However, we did not detect such differences in $lacUV5$ as a function of upstream DNA length in footprints with wild-type RNAP using hydroxyl radicals or $KMnO_4$ as probes (Figs. 11 and 12). Differences in the behavior of λP_R and $lacUV5$ could reflect the structures of the complexes formed by the two promoters or the locations of the residues potentially reactive with $KMnO_4$.

Although UP elements have been implicated in isomerization previously (12, 29, 30) and some activator proteins that bind far from the transcription-start site [e.g., λcI (50, 51) and CRP (52)] also increase k_2, the mechanism by which αCTD–DNA interactions or upstream-bound activator proteins increase isomerization step(s) remains a major unresolved issue in the mechanism of gene expression. The companion article (36) to this article suggests that the entry of downstream DNA into the "jaws" of the enzyme is affected by upstream DNA interactions with αCTD at λP_R (36), although the molecular basis for this effect is not yet understood. In any case, the data reported here indicate that αCTD–DNA interactions play a major role in the rate of association of RNAP, and we suggest that this role, although amplified by activators and UP elements, may be a general property of the initiation process at all promoters.

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