Escherichia coli RNA Polymerase Contacts outside the −10 Promoter Element Are Not Essential for Promoter Melting*

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We examined the relative affinity of model promoter constructs for binding Escherichia coli RNA polymerase (RNAP) holoenzyme. Model promoter constructs were designed to mimic DNA structures characteristic for different steps of transcription initiation. DNA duplexes in which a chemical cross-link was introduced just downstream from −10 hexamer to prevent DNA melting upon RNAP binding were used to mimic RNAP-promoter contacts in a closed complex. Fork junction DNA molecules with double-stranded/single-stranded junction between −11 and −10 position were used to study interactions of RNA polymerase with DNA in open complex. The −35 and −10 promoter regions were found to be equally important for the initial RNAP binding. The recognition of −35 promoter region was independent of structural context of the model promoter fragment. In contrast, free energy of RNAP binding to −10 hexamer was highly dependent on DNA structure. The relative importance of −10 region for sequence-specific interaction with the polymerase was the lowest for constructs mimicking closed complex and the highest for the constructs mimicking open complex. The relative importance of region −10 was also dependent on the presence of −35 consensus element indicating a communication between different DNA binding determinants of polymerase during open complex formation. Short double-stranded promoter fragments comprising only −35 and −10 or only −10 consensus elements underwent melting in a complex with polymerase indicating that the core of promoter melting activity of the polymerase is localized to a very small subset of all promoter–polymerase contacts.

Transcription is a first event of gene expression and a step, which is subjected to tight cellular control. Proper initiation of transcription is ensured by selectivity of RNA polymerase (RNAP)³ holoenzyme toward promoter DNA (1, 2). Bacterial RNAP holoenzymes are multisubunit proteins composed of core enzyme with subunit composition of α₂, β, β’, α, and one of several σ subunits (3, 4). Under normal growth conditions Escherichia coli utilizes σ³ to transcribe its genome (5, 6). Core enzyme is catalytically active, but it is incapable of initiating transcription as it displays strong but unspecific DNA binding activity (1–3). Promoter recognition and transcription initiation requires binding of σ factor to the core enzyme. Binding of σ⁰ to core enzyme induces large conformational changes of σ that are necessary for “unmasking” its DNA binding activities (7, 8). Also conformation of the core enzyme is significantly changed upon binding of σ (9, 10). Most bacterial promoters are defined by three core elements: two highly conserved hexamers located around −35 bp (−35 box; consensus sequence, TTGACA) and −10 bp (−10 box; consensus sequence, TATAAT) from the transcription start point and the linker DNA of conserved length (but not sequence) that connects the two conserved hexamers. The optimal length of this linker DNA is 17 bp (11).

Initiation of transcription is a multistep process (Refs. 2 and 12; Reaction 1). The initial binding of RNAP (R) to promoter (P) involves formation of a labile closed complex (RP₁), which can isomerize to a more stable complex (RP₅) in which promoter DNA still remains double-stranded. Isomerization between RP₅ and RP₂ complexes is likely to be rate-limiting at least in some promoters and involves major expansion of polymerase-DNA contacts and likely involves major conformational changes in the polymerase (12). Further isomerization of polymerase-promoter complex results in formation of open complex (RP₀) in which DNA duplex between positions −11 and +4 is melted. Existence of two open complexes (RP₀ and RP₅) has been suggested (12). Conversion of RP₀ to RP₅ required uptake of Mg²⁺ and involved expansion of single-stranded region to the start point of transcription. Upon addition of ribonucleotide triphosphates, RNAP in open complex starts synthesis of the first phosphodiester bond. This initiated complex (RPᵢnd) can either reverse to open complex by releasing short (<9 bp) abortive products or can undergo transition to the elongation complex. A remarkable feature of the process illustrated by Reaction 1 is that all the steps up to and including melting of promoter DNA are accomplished by the enzyme without the use of an external source of energy such as, for example, hydrolysis of ATP. The energy to form the open complex must thus come from noncovalent protein-DNA interactions between the enzyme and promoter DNA. Understanding the energetics of polymerase-promoter interactions in different intermediates of initiation reaction will be essential for understanding the mechanism of promoter melting.

Short DNA fragments containing core promoter elements in a fork junction or single-stranded conformation have become a powerful tool for dissecting polymerase-promoter interactions (13–24). Biological relevance of the results obtained using these short promoter fragments has been established by results showing that interactions with these
short promoter sequences are affected by mutations in RNA polymerase in a manner similar to interactions with whole promoters (13, 14, 17). Tsujikawa et al. (24) used fork junction DNA to show that binding of these constructs to the holoenzyme, similar to formation of open complexes with native promoter DNA, involves two kinetically significant intermediates. This further validated biological significance of using fork junction constructs as models for emulating interactions on the pathway to the open complex. Promoter fragments containing −10 region of the promoter in a fork junction conformation have been used in the past to probe polymerase-promoter interactions (15–21), to probe the mobility of critical bases in polymerase-promoter complex (22), and to obtain co-crystals of polymerase-DNA complex (23). Gralla’s laboratory has pioneered the use of such constructs, and using them provided important insights into the role of promoter-DNA contacts in the formation of the open complex. The data obtained included the initial evidence for the role of DNA fork junction binding activity in promoter opening (15, 16) and the role of various σ residues in duplex and fork junction DNA binding (17–21). The evidence suggesting a quite complex array of polymerase-promoter contacts involving both favorable and unfavorable interactions was obtained from studies in which a panel of holoenzyme mutants was tested against a panel of fork junction DNA mutants (20). Studies of the effect of base mutations in the fork junction DNA on the sensitivity of polymerase-DNA complexes to heparin challenge suggested an important role of specific polymerase-consensus −10 region interactions in promoting functionally important enzyme isomerization (18).

σ subunit plays a central role in promoter recognition and promoter melting by RNA polymerase. All sequence-specific interactions between core promoter DNA and the polymerase are presumed to occur through the σ subunit. During initiation of transcription, the −10 promoter region is presented differentially to σ subunit: first in a duplex form and then as a part of transcription bubble with almost the entire −10 sequence (downstream from −11/12 bp) in a single-stranded form (25). Sequence-specific interactions occur not only between σ and −10 hexamer in a duplex form but also persist upon melting of this region in the open complex. This conclusion was based on observations that σ subunit in the holoenzyme can bind, in a sequence-specific manner, nontemplate strand oligonucleotides corresponding to −10 box (13, 14) and fork junction DNA molecules containing at least −11A of the non-template strand in a single-stranded form (15). Region −35 remains double-stranded throughout transcription initiation, but the mode of its recognition can potentially change upon open complex formation when DNA is wrapped around RNAP and distorted from the normal linear trajectory (26).

σ-directed sequence-specific interactions between polymerase and promoter DNA in ss form could be an important source of energy necessary to melt DNA duplex. In an attempt to estimate the relative importance of these interactions for melting promoter we used simple model promoter DNA constructs mimicking polymerase-σ interactions in closed and open complexes to obtain a quantitative description of differences between the recognition of core promoter elements in ss and ds form. We observed a strong preference and higher relative sequence specificity of polymerase for binding to −10 promoter region in ss form. We furthermore observed that short ds promoter fragments containing only −35 and −10 consensus elements or −10 promoter element alone underwent melting in a complex with polymerase. All of these data point to the important role of ssDNA-polymerase interactions for promoter melting. It has been reported recently that a small fragment of the holoenzyme containing σ subunit bound to an approx. 300 amino acid fragment of the B′ subunit can efficiently melt promoter DNA (27). These data together with observations reported here provide strong evidence that promoter melting activity of polymerase is localized in a small domain of the enzyme and that polymerase-DNA contacts outside consensus −10 element are not essential for polymerase-induced promoter melting.

**EXPERIMENTAL PROCEDURES**

Materials—Cy5 monosuccinimidyl ester was purchased from Amersham Biosciences, and 4-dimethylaminobenzene-4′-sulfonyl (dabsyl) chloride was from Molecular Probes (Eugene, OR). Diethylamino-tetraacetic acid 7-amino-4-methylcoumarin maleimide (DTPA-AMCA maleimide, a luminescence donor) was prepared in our laboratory as described previously (28). The reagents for oligonucleotide synthesis and Spacer 9 phosphoramidate were purchased from Glen Research (Sterling, VA). Amine-VN phosphoramidate was from Clontech. Core RNAP enzyme was purified from E. coli K12 cells (obtained from fermentation facility at University of Alabama) using the method of Burgess and Jendrisak (29) as modified by Polyakov et al. (30) and Hager et al. (31). All the other reagents were of highest purity commercially available.

Oligonucleotides—The oligonucleotides were synthesized in our laboratory on an Applied Biosystems model 394 DNA synthesizer (Foster City, CA) using standard phosphoramidite chemistry or were purchased from commercial sources. Amino-containing oligonucleotides were labeled with N-hydroxysuccinimide esters of the dyes using standard procedures (32). Oligonucleotides in trityl-on form and oligonucleotides labeled with fluorescence probes were purified using reverse phase chromatography as described previously (32) or by denaturing polyacrylamide gel electrophoresis. Purity of oligonucleotides after purification was checked by 8 M urea denaturing gel electrophoresis followed by ethidium bromide staining. Oligonucleotides were 95–99% pure as determined by this assay. Duplex DNA promoter fragments were formed by mixing equimolar amounts of appropriate complementary strands in a buffer containing 50 mM HEPES (pH 7.9), 100 mM KCl, 10 mM EDTA; heating for 2 min at 95°C; and slowly cooling down to 25°C over 2 h. Hybridization of duplexes was confirmed by native gel electrophoresis.

The sequence of oligonucleotides used in this study was derived from λp 35 promoter sequence (position −38 to −4 bp) (33) with the following changes: (i) −35 box and −10 box sequences were changed to match the consensus sequences: TTGACT to TTGACA and GATAAT to TATAAT, respectively, and (ii) a point mutation at position −14 (G to T) was incorporated to reduce similarity to the extended −10 sequence. The following oligonucleotides were used in this study (−10 and −35 sequences if present are underlined): AN1, GTG TTT ACA ATT TTA CCT CTG GCG GTT ATA ATG GT; AN2, TAA CCA (amine-VN) CCA GAG GTA AAA TTG TCA ACA C; AN3, GTG TTT ACA ATT TTA CCT CTG GCG TTG ATA ATG GT; AN4, TAA ACG CCA GAG GTA AAA TTG TCA ACA C; AN5, GTG TTT ACA ATT TTA CCT CTG GCG TTT ATG G(S9) C AT CAT TAT AAA CCG CAG AGG TAA AAT TGT TCA ACA C; AN6, ACC ATT ATA AAC GCC AGA GGT AAA ATG GTC AAC AC; and AN7, GCC GTT TAT AAT GG. S9 corresponds to Spacer 9 linker (Glen Research) and amine-VN is a phosphoramidate-containing amino group linked to a sugar backbone (Clontech).

Hybridization of Cy5-labeled AN2 with AN1 resulted in a reference fork that produced luminescence resonance energy transfer (LRET) signal when bound to europium chelate-labeled RNAP. This complex was used in all competition experiments with various unlabeled DNA constructs described in the text. AN3/AN4 produced long fork constructs
containing both −35 and −10 regions, AN5 produced a DNA hairpin (with DNA strands downstream of the −10 region linked bySpacer 9) containing both −10 and −35 regions, AN3/AN6 produced a duplex containing both −10 and −35 regions, and AN7 is a short ssDNA corresponding to non-template strand containing −10 region. In addition to the oligonucleotides listed above, a number of oligonucleotides of analogous structure but with various mutations incorporated into −10 and/or −35 regions were also used (as described in the text). The structures of various DNA constructs used in this work are schematically illustrated in Fig. 1.

Preparation of RNAP Holoenzyme Containing a Subunit Labeled Specifically with (Eu3+)-DTPA-AMCA—A single cysteine mutant of σ70 ((A59C)σ70) was overexpressed in E. coli cells, purified, and labeled with thiol-reactive fluorochrome DTPA-AMCA maleimide as described previously (8). This mutant and its derivative labeled with Eu3+ chelate had properties very similar to the wt protein as determined by transcription activity assays, core polymerase binding assays, and open complex formation assays using full promoter sequences (data not shown). An equimolar amount of EuCl3 was added to a solution of DTPA-AMCA-labeled (A59C)σ70. RNAP holoenzyme was reconstituted from core enzyme and modified σ70 and purified from an excess of free σ70 on a Superdex 200 HR sizing column (Amersham Biosciences) as described elsewhere (8).

Equilibrium Competition Binding Assay Using LRET—Relative affinities of RNAP holoenzyme toward different unlabeled promoter DNA fragments were determined by measuring the ability of these molecules to compete with Cy5-labeled reference fork junction promoter fragment (AN1/AN2). An equilibrium competition assay was performed in a final volume of 120 μl at 25 °C in 50 mM Hepes (pH 7.9), 250 mM KCl, 10 μM EDTA, 10 mM MgCl2, 1 mM dithiobthreitol, 0.1 mg/ml bovine serum albumin, 2.8% polyethylene glycol (8000). Polyethylene glycol was used as a molecular crowding agent. It improves the quality of the data without any negative effect on RNA polymerase. The final concentrations of Cy5-labeled reference fork DNA and RNAP holoenzyme containing (A59C)σ70 labeled with (Eu3+)DTPA-AMCA were 25 and 10 nM, respectively. In some experiments (for equilibrium competition with single-stranded DNA constructs containing −10 region only, oligonucleotide AN7, and its derivatives), Cy5-labeled single-stranded oligonucleotide containing nontemplate −10 sequence was used as reference probe, and the final concentrations of acceptor-labeled DNA and donor-labeled RNA polymerase were 100 and 20 nM, respectively. The concentration of unlabeled competitor promoter fragments was in the nanomolar to micromolar range depending on the relative affinity of the competitor for RNA polymerase holoenzyme. RNAP was added to the reaction mixture containing reference labeled DNA and unlabeled competitor DNA, and after a 5-min incubation at 25 °C, sensitized acceptor emission at 668 nm was determined using pulse nitrogen laser excitation at 337 nm. Sensitized acceptor emission and donor emission (collected at 620 nm) were measured simultaneously on a laboratory-built two-channel instrument described earlier (34). The time-resolved sensitized acceptor signal resulting from 1000 laser pulses was accumulated. Total sensitized acceptor signal was calculated and normalized as described previously (35). The relative affinities of competitor DNA were determined from the measurements of the relative degree of saturation of RNA polymerase with Cy5-labeled reference fork junction molecule and unlabeled competitor DNA as described previously (35) using Equations 1–4,

\[ \Delta \Delta G_{\text{ref-comp}} = \Delta G_{\text{ref}} - \Delta G_{\text{comp}} = -RT \cdot \ln \left( \frac{K_{\text{comp}}}{K_{\text{ref}}} \right) \]  

(Eq. 1)

\[ K_{\text{comp}}/K_{\text{ref}} = \left( \frac{\nu_{\text{comp}}(\text{DNA}_{\text{ref}})_{\text{tot}} - \nu_{\text{comp}}(\text{RNAP})_{\text{tot}}}{\nu_{\text{ref}}(\text{DNA}_{\text{comp}})_{\text{tot}} - \nu_{\text{comp}}(\text{RNAP})_{\text{tot}}} \right) \]  

(Eq. 2)

\[ \nu_{\text{ref}} = \frac{F_{\text{comp}}}{F_0} \]  

(Eq. 3)

\[ \nu_{\text{comp}} = 1 - \nu_{\text{ref}} \]  

(Eq. 4)

where \( \Delta \Delta G_{\text{ref-comp}} \) is a free energy difference between the affinity of the competitor and reference DNA; \( \nu_{\text{ref}} \) and \( \nu_{\text{comp}} \) are the degrees of saturation of RNAP with reference Cy5-labeled DNA and competitor unlabeled DNA, respectively; \( [\text{DNA}]_{\text{ref}} \) and \( [\text{DNA}]_{\text{comp}} \) are the total concentrations of reference Cy5-labeled DNA and competitor unlabeled DNA, respectively; \( [\text{RNAP}]_{\text{tot}} \) is the total concentration of RNAP; \( F_0 \) and \( F_{\text{comp}} \) are sensitized acceptor emissions in the absence and presence of unlabeled competitor DNA fragment, respectively.

Fluorescence Assay for Melting of Short Promoter Fragments—Fluorescence spectra and time-dependent fluorescence changes of fluorescein and dabcyl-labeled promoter fragments were recorded using an Amino Bowman Series 2 spectrofluorometer (Spectronic Instruments, Rochester, NY) with excitation at 490 nm and emission at 520 nm. Fluorescence spectra of promoter fragments at various concentrations of the enzyme were obtained using 35 nM labeled DNA fragments incubated with the indicated concentrations of the holoenzyme for 15 min at 25 °C. Specificity of fluorescence increase upon addition of RNA polymerase holoenzyme to fluorescein/dabcyl-labeled double-stranded DNA was confirmed in a competition assay. Holoenzyme in a final concentration of 105 nM was added to the reaction mixture containing 35 nM labeled ds promoter fragment and 420 nM unlabeled ds promoter fragment with exactly the same sequence or 420 nM ds promoter fragment with mutated sequences in −35 and −10 hexamers. After a 15-min incubation at 25 °C fluorescence emission spectra were collected by scanning from 500 to 650 nm.

Fluorescence increase upon addition of RNA polymerase holoenzyme to fluorescein- and dabcyl-labeled double-stranded DNA as a
function of time and temperature was measured by recording the time trace of fluorescence emission (at 520 nm) upon addition of RNAP holoenzyme at a final concentration of 210 nM to the reaction mixture containing 35 nM labeled DNA preincubated at 14, 25, or 37 °C. The curves were analyzed using SCIENTIST (Micromath, Salt Lake City, UT) according to a single exponential kinetic equation (Equation 5),

\[ F = A \cdot \{1 - \exp(-k \cdot t)\} + C \]  

(Eq. 5)

where \( F \) is the observed fluorescence of fluorescein at time \( t \); \( A \) and \( k \) are the amplitude and observed rate constant, respectively; and \( C \) is the fluorescence intensity at time \( t = 0 \).

**Potassium Permanganate Sensitivity Assay**—Potassium permanganate footprinting was performed on a fully double-stranded 35-bp promoter DNA fragment (−38 to −4) containing consensus −10 and −35 sequences and labeled with fluorescein on the template strand. Holoenzyme (1.5 μM) containing a wt or or melting-deficient mutant (FYW mutant (36) in which aromatic residues Phe-427, Tyr-430, and Trp-433 and incubation was continued for 35 min at three different temperatures (14, 25, and 37 °C) in a 25 μl final volume. Samples containing no enzyme or core enzyme were used as negative controls. Fluorescein-labeled single-stranded template strand was used as a positive control. At the end of the incubation heparin was added to all samples to 0.2 mg/ml final concentration, and after a 5-min incubation, KMnO₄ was added to 15 mM concentration. The reaction was carried out for 5 min and was stopped by the addition of a mixture containing β-mercaptoethanol (to 1.5 mM), glycogen (to 0.8 mg/ml), and a reference fluorescein-labeled 50-base long ssDNA fragment (to 0.14 μM). 100 μl of H₂O was added, and samples were extracted with 1 volume of phenol (equilibrated with 10 mM Tris (pH 7.4), 1 mM EDTA buffer) followed by 1 volume of chloroform. DNA was precipitated by addition of 30% volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold ethanol. The DNA pellet was washed with 750 μl of 70% ethanol, dried, dissolved in 100 μl of 1 M piperidine, and incubated for 30 min at 90 °C. Samples were vacuum-dried, washed in 1 ml of H₂O, and vacuum-dried overnight to remove piperidine. The pellet was dissolved in 5 μl of 95% formamide, 20 mM EDTA and incubated for 2 min at 90 °C. Samples were loaded on a denaturing 7 M urea, 18% polyacrylamide gel (16 × 14 × 0.75 cm; acrylamide:bisacrylamide = 19:1). The gel was run in 1× TBE (90 mM Tris borate, 2 mM EDTA) at 25 °C for 1 h 50 min at 450 V (a prerun was performed for 1 h at 300 V) in a model SE 400 electrophoresis box (Hoefer Scientific Instruments, San Francisco, CA). DNA bands labeled with fluorescein were detected in the wet gel using a STORM fluoroiimag (Amersham Biosciences) using blue mode scanning. Bands intensities were quantified using Image Quant (Amersham Biosciences).

**Competitive Electrophoretic Mobility Shift Assay (EMSA)**—25–40 nM RNAP was added to a solution containing 25–40 nM Cy5-labeled reference fork junction molecule (AN1/AN2) and varying amounts of unlabeled competitor promoter fragment constructs in 50 mM HEPES (pH 7.9) buffer containing 250 mM KCl, 10 μM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 2.8% polyethylene glycol (8000). Samples (10 μl) were incubated at 25 °C for 5 min, and RNAP-DNA complexes were resolved from free DNA by electrophoresis on a native 4% polyacrylamide gel (19:1 acrylamide:bisacrylamide) running in 1× TBE at 25 °C for 1 h at 80 V. Bands containing Cy5-labeled DNA were detected using a STORM fluoroiimag (Amersham Biosciences) using red mode scanning. Bands intensities were quantified using Image Quant (Amersham Biosciences).

**RESULTS**

Fork junction DNA has been established as a very useful model mimicking interactions of RNA polymerase with promoter DNA (15). Because the bacterial RNAP is able to bind and melt promoter DNA without the assistance of auxiliary factors or an external source of energy (such as, for example, ATP hydrolysis), noncovalent interactions between polymerase and promoter DNA must provide the driving force to guide polymerase through all intermediates of transcription initiation reaction. The major goal of the studies presented here was to elucidate relative contributions of various protein-DNA contacts between RNAP and model promoter fragments to the overall stability of the complex and to probe into the role of these contacts in the formation of the open complex.

**Relative Contributions of −35 and −10 Elements to the Stability of RNAP-Fork DNA Complex**—To measure relative affinity of various promoter constructs for binding RNA polymerase holoenzyme, we adopted the luminescence energy transfer-based competition assay described previously (35). This assay allows determination of relative affinity (ΔΔG) of the construct of interest compared with a reference fluorophore-labeled ligand even when the binding itself is too tight to be measured quantitatively. RNA polymerase holoenzyme was reconstituted using σ subunit single reactive cysteine mutant modified with europium chelate luminescence donor (28). Binding of Cy5-labeled reference DNA construct results in a protein-DNA complex producing large sensitized emission of Cy5 acceptor when the donor europium chelate was excited (Fig. 2A). Using pulsed excitation and time-gated acceptor emission measurement, low levels of background signal can be achieved (Fig. 2B and Ref. 37) because background scattering and prompt emission of directly excited acceptor molecules decay to zero shortly after the excitation pulse is discontinued (37). In all measurements we used a reference construct a fork junction DNA construct obtained by hybridization of AN1 with Cy5-labeled AN2. This construct contained consensus −35 and consensus −10 boxes, contained Cy5 label at position −16 of the template strand, was double-stranded from position −38 to −11, and contained a single-stranded nontemplate strand extension from −10 to −4. When the unlabeled competitor was present, the decrease of sensitized acceptor signal intensity could be used to determine relative affinity of the competitor using the formula described previously (35). For example, Fig. 2B shows that in the presence of equimolar amounts of the unlabeled reference construct an ~40% decrease of acceptor emission was observed indicating slightly higher affinity of Cy5-labeled reference construct compared with unlabeled reference construct. Direct titration of the holoenzyme with Cy5-labeled reference construct revealed tight binding with subnanomolar affinity (Fig. 2C). Similarly tight binding was revealed using electrophoretic mobility gel shift assay (38) (Fig. 2D). In this assay the complexes were challenged with heparin before loading on the gel. Similar affinities obtained in the LRET assay and EMSA assay indicated proper method-independent behavior of our constructs. Fig. 3A shows the results of the experiment in which relative binding affinities were determined for three different constructs containing the same consensus promoter sequence but differing in the manner by which −10 sequence was presented to the polymerase: (i) entire −10 region was double-stranded (AN3/AN6); (ii) entire −10 was double-stranded, and top and bottom strands were covalently cross-linked at −4 to prevent melting of DNA duplex (hairpin AN5 construct); and (iii) −10 region was in fork junction DNA arrangement with the last bp of the duplex at −11 and nontemplate strand in single-stranded form extending to −4 (AN3/AN4) (Fig. 1). These measurements revealed a large (~2 kcal/mol) preference of the holoenzyme for binding fork junc-
A competition binding assay using LRET. A, design of the assay. Binding of acceptor (Cy5)-labeled fork junction DNA to donor (europium chelate)-labeled RNAP brings both fluorophores to relative proximity producing a large sensitized acceptor LRET signal ($F_\text{comp}$). In the presence of unlabeled competitor DNA, the dissociation of labeled fork junction DNA will reflect the relative affinity of the unlabeled competitor. The degree of competition is determined by measuring sensitized acceptor LRET signal in the presence of competitor DNA ($F_\text{comp}^\text{C}$) and comparing it with $F_\text{o}$. B, typical sensitized signals and background of the assay: bar 1, donor-labeled $\sigma^{70}$ alone (10 nM); bar 2, donor-labeled $\sigma^{70}$ (10 nM) in the presence of Cy5-labeled fork junction DNA (25 nM); bar 3, donor-labeled RNAP alone (10 nM); bars 4–6, donor-labeled RNAP in the presence of unlabeled constrained DNA molecule (bar 4), Cy5-labeled fork junction DNA (bar 5), or an equimolar mixture of Cy5-labeled and unlabeled fork junction DNA (bar 6). C, saturation of RNA polymerase holoenzyme with Cy5-labeled fork junction DNA measured by EMSA. Fork junction DNA (25 nM) was incubated with increasing amounts of RNAP at 25 °C for 5 min and challenged with 100 μg/ml heparin for 3 min. Reaction mixtures were resolved by native 4% PAGE, and fluorescence intensity of bands corresponding to the complex (inset) was determined using a STORM PhosphorImager using red laser excitation. The solid line represents the nonlinear regression fit of the data to an equation describing a simple binary complex formation.

FIGURE 3. Relative affinity of DNA constructs. A, relative affinity of fork junction DNA construct (black bar), hairpin DNA construct (light gray bar), and duplex DNA construct (dark gray bar). All constructs contained consensus −10 and −35 elements, and the relative affinity ($\Delta G$) reflects differences in affinity between Cy5-labeled fork junction DNA containing consensus −10 and −35 elements and the indicated construct. B, structural context dependence of the relative affinity of DNA constructs in which entire −10 or −35 regions were eliminated by replacing consensus bases with their corresponding purine (or pyrimidine) counterparts. Black bars, fork junction DNA constructs; light gray bars, hairpin DNA constructs; dark gray bars, duplex DNA constructs. Relative affinity ($\Delta G$) reflects differences in affinity between constructs containing consensus −10 and −35 elements and constructs of the same overall structure but containing the indicated mutations of −10 and/or −35 elements.

Relative affinity ($\Delta G$) reflects differences in affinity between constructs containing consensus −10 and −35 elements to evaluate relative sequence-specific contributions (and their dependence on structural context) of −10 and −35 hexamers to the overall binding affinity. We thus compared the effects of removing conserved −10 and −35 hexamers within the context of the constructs used in experiments illustrated in Fig. 3A on the binding affinity of the constructs (Fig. 3B). To eliminate the consensus −10 or −35 hexamer, all conserved nucleotides in the hexamer were mutated to their purine or pyrimidine counterparts (i.e. T→C, A→G, etc.) to create “anti-consensus” hexamers. Elimination of −35 hexamer had a very modest (<1 kcal/mol) and essentially equal effect on binding affinity in all three DNA constructs. In contrast, the effect of mutating −10 region greatly depended on the structural context of the construct. Mutation of the
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![FIGURE 4. The effect of single base substitutions on the relative affinity of fork junction DNA constructs. A, substitutions in −35 region. The label above each bar denotes the base substitution. All other bases in −35 region and all bases in −10 region were consensus. B, substitutions in −10 region. The label above each bar denotes the base substitution. All other bases in −10 region and all bases in −35 region were consensus. Relative affinity of the constructs (ΔΔG) reflect differences in affinity between fork junction DNA construct containing consensus −10 and −35 elements and fork junction DNA constructs containing the indicated single base substitutions.](image)

![FIGURE 5. Context dependence of the effect of substitutions in −10 region. Relative affinity of fork junction DNA constructs containing the substitutions indicated by the labels above the bars was measured in a context of fork junction DNA containing consensus −35 region (black bars) or a construct containing anti-consensus −35 region, i.e. where all consensus bases were replaced with their corresponding purine (or pyrimidine) counterparts (gray bars). Relative affinity of the constructs (ΔΔG) reflects a difference in binding energy between the mutant indicated by the label and the analogous construct containing wt −10 region.](image)

The entire −10 box in fork junction DNA construct resulted in an ∼3 kcal/mol loss of binding energy, whereas less than 1 kcal/mol was lost when the same mutations were introduced into the construct with top and bottom strands covalently cross-linked at −4 position (hairpin construct). The loss observed in the context of fully double-stranded construct was ∼2 kcal/mol. The pattern of these relative affinities of the holoenzyme appears to be well suited to guide polymerase from the initial complex with the promoter in ds form to the final open complex containing region from approximately −11 to approximately +2 in single-stranded form. The DNA construct in which top and bottom strands were covalently cross-linked at −4 position should most closely mimic interactions with double-stranded promoter DNA. The interstrand cross-link at position −4 should prohibit melting of DNA duplex in −10 region. Interaction of the polymerase with this construct is characterized by relatively low affinity and low sequence specificity. Within this construct, sequence-specific contributions of −10 and −35 regions appear to be small and essentially equal. This would indicate that sequence-specific contributions of −10 and −35 regions for the initial polymerase recognition are comparable. When −10 region was in fork junction DNA conformation, the loss of binding energy due to −10 region mutation was much larger. Thus, −10 region, when partially single-stranded, is recognized by polymerase not only with higher affinity and but also with higher specificity. This suggests that once melting of promoter DNA is initiated (for example, through spontaneous or polymerase-catalyzed base or DNA segment flipping out), a sizable thermodynamic driving force to complete the promoter melting reaction will be available as a result of this preferential binding of polymerase to DNA conformation corresponding to the end point of the melting reaction. The effects of −10 and −35 consensus hexamer mutations observed in the case of the duplex construct were somewhat bigger compared with cross-linked duplex construct (Fig. 3B). This is most likely because of partial melting of this construct upon binding to the polymerase (see below).

![Image](image)

Context Dependence of the Effect of Individual Substitutions within −10 Box—The data summarized in Fig. 4B are in contrast to a large decrease of binding energy observed when all −10 element positions were mutated (Fig. 3B). This is also in contrast with our previous data obtained with short fork DNA construct containing region −10 only (35). These data showed that individual mutations at positions −11 and −7 resulted in a large decrease in binding affinity, whereas individual mutations at positions −12, −10, −9, and −8 produced small or no effects. Because the obvious difference between the experiments illustrated in Fig. 4B and our previous data were the length of the construct and the presence or absence of −35 box, we compared the effects of individual mutations within −10 hexamer in a context of a long fork junction DNA construct (−38 to −4) containing either consensus −35 box or anti-consensus −35 box (with all six bases within −35 box mutated). As shown in Fig. 5, the results of this comparison were very striking. The effects of individual mutations within −10 box on binding affinity greatly depended on the presence or absence of a functional −35 box. As described above, in its presence, individual mutations in −10 box had a very small effect on binding affinity. In contrast, in its absence, individual mutations at positions −11 and −7 produced a large decrease in binding affinity in agreement with the results obtained previously using short fork junction DNA construct (−26 position to −4) containing only −10 box (35) and in agreement with the effect of these mutations on the affinity of short single-stranded oligonucleotides containing non-template −10 region sequence (AN7 oligonucleotide and its derivatives; Fig. 1 and data not shown). To further verify context dependence of the effects of individual substitutions within −10 region (and prove that these results were not dependent on the methodology used to determine binding), we used a competitive EMSA assay to assess
In these experiments, a complex of the polymerase with Cy5-labeled fork DNA construct containing consensus −35 and −10 boxes was incubated with increasing concentrations of unlabeled competitor constructs. Reaction mixtures were then loaded on a native polyacrylamide gel, and the relative amount of Cy5-labeled complex remaining at each competitor concentration was measured by determining the relative fluorescence intensity of a band on the gel corresponding to a complex. These data were plotted (Fig. 6A) and fitted to a simple competition binding scheme to obtain apparent binding constants for the various constructs. The results obtained using competition EMSA were in complete agreement with the data obtained using the LRET assay described above. The same order of relative affinities of various constructs was observed (i.e. fork DNA was the best binder followed by hairpin and duplex constructs). The sequence-specific component of binding affinity was the highest for the fork DNA construct. Most significantly, the dependence of the effects of individual mutations in −10 box on the presence or absence of a functional −35 box was also evident (Fig. 6B). Taken together, the results shown in Figs. 5 and 6 indicate an intriguing and previously unrecognized functional communication between −35 and −10 box interactions with the polymerase.

**Melting of Minimal Promoter Fragments**—Models of promoter melting by RNA polymerase often implicated an essential role for polymerase-promoter contacts upstream and downstream of the core promoter elements (25, 39). A significant thermodynamic driving force for melting promoter DNA uncovered by binding experiments involving minimal model promoter DNA fragments described above raised the question of whether this driving force could be sufficient for polymerase-induced DNA melting. To address this question we prepared a fully double-stranded minimal promoter DNA construct containing only the core −35 and −10 promoter elements (−38 to −4) designed to report promoter melting by fluorescence intensity change (Fig. 7A). This construct thus lacked promoter regions involved in upstream and downstream contacts with the polymerase (for example, it did not contain promoter regions involved in contacts in the vicinity of the active site of the enzyme). We introduced the fluorescence probe (fluorescein) to the
3′-end of the nontemplate strand and the quencher (dabcyl) to the 5′-end of the template strand. In a duplex, fluorescein and dabcyl were in close proximity resulting in efficient quenching of fluorescein emission. We expected that if this construct would undergo melting, the distance between fluorochrome and the quencher would increase, and a large increase of fluorescence intensity should be observed. This is exactly what we observed. Upon mixing of polymerase with this reporter construct a large increase in fluorescence intensity was observed (Fig. 7B). This increase had to be due to the increased separation between fluorochrome and quencher because only a minor change of the intensity was observed with DNA construct that was labeled only with fluorescein (not shown). The observed fluorescence increase was dependent on polymerase concentration and reached saturation at nanomolar protein concentrations (Fig. 7C). The kinetics of fluorescence intensity changes following mixing of polymerase with the reporter construct appeared to involve a rapid phase occurring within the mixing time of the sample followed by slower phase (Fig. 7D). The simplest interpretation of these signal changes would be to assume that the fast change corresponded to an initial binding of the polymerase to the promoter fragment followed by a slower DNA melting step. The rate of the slower step increased greatly with the increase in temperature (not shown), which is the behavior expected for a step involving melting of DNA duplex. Mutant construct with a single base substitution at position −11 exhibited a greatly reduced time-dependent fluorescence increase upon addition of the polymerase (Fig. 7D) consistent with the presumed important role of this position in promoter melting. To confirm that fluorescence signal increase observed upon addition of the polymerase was indeed due to melting of the DNA fragment, the same construct as used in the fluorescence experiments was probed by permanganate footprinting (40). Hyperreactivity of template strand thymines at positions −11, −9, and −8 was clearly visible (Fig. 8) upon binding of the construct to RNAP. The effect was specific to the −10 region because no reactivity of thymines at various positions upstream of −10 box was observed. The effect was strictly dependent on σ subunit because no increase of thymine reactivity was observed when core enzyme alone was used. Also σ mutant with previously established melting deficiency (FYW mutant (36)) was also deficient in inducing hyperreactivity of thymines in −10 box. Based on the data illustrated in Figs. 7 and 8 we concluded that minimal promoter fragment containing only −35 and −10 boxes and lacking promoter regions making extensive upstream and downstream contacts with the polymerase was efficiently melted upon binding to the enzyme.

This efficient melting of minimal promoter fragment prompted us to investigate whether even a smaller promoter fragment could undergo melting upon binding to the polymerase. We prepared a short DNA duplex containing only −10 box (−18 to −5). Permanganate footprinting revealed that this fragment was also melted upon binding to the polymerase although with significantly lesser efficiency compared with the longer promoter fragment used in the experiments illustrated in Fig. 8. In contrast to the longer promoter fragment, detection of the melting of short −10 only duplex required elevated temperatures (it could not be detected at 14 °C). Melting was strictly dependent on σ subunit (no detectable melting in the presence of the core enzyme) and was impaired when melting-deficient mutants of σ were used (Fig. 9). Taken together, the data illustrated in Figs. 8 and 9 show that only a small subset of all possible polymerase-promoter contacts is necessary for promoter melting activity of the polymerase.

**DISCUSSION**

Data presented in this study add two new significant insights into the role of −10 region-promoter interactions. First it appears that a sizable amount of favorable free energy to facilitate promoter melting is available to RNA polymerase as a result of preferable interactions of σ subunit with the nontemplate strand of −10 region. That this is an important driving force for promoter melting is evident from the data showing that a very short promoter fragment containing region −10 only undergoes melting upon binding to RNA polymerase. This indicates that although polymerase contacts outside consensus −10 region can play important roles in facilitating open complex formation, the core of promoter melting activity is localized to a very small subset of all promoter-promoter contacts. Recently Young et al. (27) have shown that a surprisingly small fragment (~20%) of RNA polymerase holoenzyme was capable of forming an open complex. In this fragment, most of polymerase elements thought to participate in promoter-polymerase
contacts outside conserved core promoter elements were missing. Our data are consistent with and further reinforce these observations and the conclusions derived from these observations.

A second new feature of polymerase-promoter contacts that is apparent from the data presented here is the communication between the contacts that polymerase makes with −10 and −35 consensus regions. This is most clearly demonstrated by the striking dependence of the consequences of mutating the bases at position −11 or −7 on the presence or absence of a functional −35 element. When this element is present, mutating these bases often has a very small effect on the binding affinity of fork DNA constructs (Figs. 5 and 6). When this element was replaced with unrelated DNA, mutations at −11 or −7 positions produced a very large decrease of binding affinity (Figs. 5 and 6). In simple words, the region of the polymerase involved in −10 element recognition "feels" the nature of contacts being made by −35 binding region. In RNA polymerase holoenzyme, structural elements making contacts with −10 and −35 elements are far apart (~50–70 Å) (9, 10), which precludes explaining the communication between these regions through direct contacts. This effect must thus involve long range communications either through the protein or through the spacer DNA connecting −10 and −35 promoter elements (or both). Large conformational changes in the polymerase have been identified by comparing various structures of the core enzyme (41) or by comparing structures of the core and the holoenzyme (9, 10, 42). Such observations point to the structural flexibility of the enzyme and its general ability to undergo conformational transitions. The crystal structure of the holoenzyme in complex with a short promoter fragment revealed conformational changes in both regions of α subunit involved in −10 and −35 promoter element recognition (region 2 and region 4, respectively). Region 2 rotated 3° closing the RNAP main channel by about 3 Å, and region 4 rotated 4° resulting in the movement of −35 recognition helix by ~6 Å (23). With currently available data, it is not possible to determine whether the above conformational changes are involved in the communication between regions of the polymerase involved in recognizing −10 and −35 promoter elements. More biochemical data will be required to understand the mechanism of this communication.

The observation of context dependence of energetic consequences of mutations in −10 region also sheds some light on discrepancies regarding these issues present in the literature. We have reported previously, using very short fork junction DNA constructs (containing only −10 region DNA), that consensus −11A and −7T provide major sequence-specific contributions to the binding energy (35). In contrast, Fenton and Gralla (18), using longer fork junction DNA that contained functional −35 region, reported only small sequence-specific contributions of consensus bases at these positions. Although there were significant differences in experimental conditions between these two studies (with probably the most important being the temperature, 25 °C in Ref. 35 and 0 °C in Ref. 18) and the methodologies used to determine binding affinity, which could explain these somewhat different results, context dependence of the effects of mutations in −10 region described above provides a much more likely explanation of these discrepancies. The next challenge will be to determine the functional role of the cross-talk between −10 and −35 binding domains of RNA polymerase.

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