A Consensus Adenine at Position −11 of the Nontemplate Strand of Bacterial Promoter Is Important for Nucleation of Promoter Melting

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Numerous studies have suggested an important role of adenine at position −11 of the nontemplate strand of bacterial promoters for sequence-specific recognition of the −10 promoter element in single-stranded form. In this work, we attempted to identify a specific step in transcription initiation reaction that is most critically dependent on specific recognition of −11A. Mutating −11A in the context of a model promoter resulted in a profound decrease of the rate of heparin-resistant promoter complex formation and in a modest increase of the rate of heparin-resistant complex dissociation. The identity of nontemplate base at position −11 became relatively unimportant when the duplex in the vicinity of this position was destabilized by base pair mismatches. For promoters with a nonnative thymine at nontemplate position −11, we observed a remarkable correlation between the rate of heparin-resistant complex formation and the free energy of duplex stability in the vicinity of this residue, indicating that the replacement of −11A with a T affected a step in the reaction that involves local melting of DNA duplex. These data show that a promoter melting defect caused by a loss of RNA polymerase contact with −11A can be rescued by artificially induced local destabilization of the DNA duplex. These results are consistent with and support the idea that specific recognition of adenine at the nontemplate −11-position is important only for the initial nucleation of melting, which probably involves the flipping of this adenine out from the DNA duplex.

The transcription initiation reaction includes a critical step of promoter melting that is needed to expose the DNA template strand for template-directed RNA synthesis. Transcription initiation in the case of bacterial RNA polymerase (RNAP) is a multistep reaction involving at least two major steps, a closed and an open complex (1–3). In the closed complex, the enzyme makes sequence-specific contacts with at least one of the two conserved DNA hexamers that define bacterial promoter (the −10 and −35 elements). DNA template remains double-stranded in the closed complex. Subsequent isomerization to the open complex involves further conformational changes in the polymerase-promoter complex and includes a crucial DNA melting reaction. It has been suggested that this transition from closed to open complex involves two steps: a conformational transition in RNAP-promoter complex and a subsequent melting of promoter DNA (1–3). There is evidence that, at least for some promoters and at physiological temperatures, promoter melting is very rapid and that the step involving conformational transition in RNAP-promoter complex is rate-limiting for open complex formation (4–7). However, significant evidence exists that suggests that some localized melting does occur during the rate-limiting step involving conformational change in RNAP. For example, extensive study of the effects of base depurination and DNA prenicking in the vicinity of the −10 region on open complex formation showed that facilitation of DNA melting contributed to the rate of open complex formation (8). Such observations are consistent with an idea that promoter melting leading to formation of the open complex is a stepwise process that can be divided into at least two steps: nucleation of melting involving a very small subset of promoter region, which eventually becomes single-stranded in the open complex, and subsequent expansion of the melted region (3). Stepwise melting has been detected in footprinting experiments (9) and can be amplified by mutations in RNAP (10) or by manipulating reaction conditions (9, 11–13).

Precise molecular mechanism of RNAP-induced promoter melting has not yet been established. Bacterial RNAP can melt promoter in the absence of any accessory factors and without the input of energy from external sources. This indicates that the enzymemelts promoter DNA utilizing the energy from favorable noncovalent protein-DNA interactions in the RNAP-promoter complex. There are numerous contacts between various subunits of RNAP and promoter DNA (1–3). However, sequence-specific and strand-specific recognition of −10 promoter sequence by the σ subunit of RNAP holoenzyme (14–23) is the most likely candidate interaction that could be the source of favorable energy to facilitate DNA melting. Base-specific recognition of the nontemplate strand of the −10 region of the promoter was first suggested by the unexpected requirement of the σ subunit for promoter-proximal pausing at the phase λ gene promoter. DNA sequences required for this pausing exhibited striking similarity to nontemplate strand −10 sequence (14). Subsequent binding studies utilizing single-stranded oligonucleotides or fork junction partial duplexes containing nontemplate strand single-stranded extensions confirmed that σ subunit in the holoenzyme recognizes the −10 nontemplate strand sequence with high selectivity and high affinity (15–18). The potential importance of σ-nontemplate −10 DNA interactions in promoter melting is highlighted by the recent findings that the core of promoter melting activity of RNAP is localized to a very small subset of all promoter-polymerase contacts, limited essentially to σ −10 nontemplate strand contacts (19, 20). The above
Nucleation of Promoter Melting

Materials—Sybr Green was purchased from Molecular Probes, Inc. (Eugene, OR). All of the other reagents were of the highest purity commercially available. Core RNAP enzyme was purified from Escherichia coli K12 cells (obtained from the fermentation facility at the University of Alabama) using the method of Burgess and Jendrisak (26) as modified by Polyakov et al. (27) and Hager et al. (28). Recombinant WT α20 was expressed and purified as described previously (29). Holoenzyme was reconstituted by incubating 2 μM core with 6 μM α20 for 15 min at 37 °C. Oligonucleotides were obtained from commercial sources (IDT in Coralville, IA) or the Keck Oligonucleotide Synthesis Facility at Yale University (New Haven, CT). Fluorescein (when present) was added to the reaction mixture containing only the –10 consensus promoter element (20).

Conclusion is based on data showing that a small fragment of RNAP (containing polymerase elements responsible for –10 nontemplate strand recognition) could efficiently melt promoter DNA (19). Furthermore, RNAP was found to melt very short promoter fragments containing only the –10 consensus promoter element (20).

Adenine at position –11 in the nontemplate strand appears to be of special importance for promoter melting reaction. It is one of the most conserved residues in the –10 region of bacterial promoters (21). It is also critically important for sequence-specific recognition of the nontemplate strand –10 region by the α subunit in the holoenzyme, since mutations at this position strongly decrease the binding affinity (16–22). Fine discrimination of determinants responsible for the preference for –11 adenine identified the N1 position of the base as critical for high affinity binding (22). Position C2 was also suggested as important for –11A function (23). However, available data suggest that the C2 position is not essential and that the effects of substitutions at C2 are likely to be indirect due to close proximity of the C2 and N1 positions (22). In the DNA duplex, the N1 position of adenine is normally involved in hydrogen bonding with its complementary T residue. The interaction of RNAP with the N1 of –11 adenine and the A-T base pairing at –11 are thus mutually exclusive, indicating that the –11A interaction with RNAP could either catalyze disruption of the –11A-T base pair or could stabilize extrahelical conformation of spontaneously flipped-out –11A. Dynamic fluorescence polarization studies indicated tight association of the –11A with the polymerase in model RNAP-DNA complexes containing –11A in single-stranded conformation, supporting the notion of a specific contact between –11A and RNAP (24). Protein-catalyzed or protein-stabilized DNA duplex states with a single base flipped out of the DNA duplex are well documented in the case of DNA repair enzymes (25). All of the above characteristics of the nontemplate –11A together with its locations exactly at the upstream boundary of the transcription bubble (where promoter melting is presumably nucleated) suggest a possibility that –11A could play a specific role in facilitating nucleation of promoter DNA melting through a flipped-out intermediate involving this residue. We describe here results of experiments indicating that indeed –11A interactions with the polymerase are involved in promoter melting nucleation.

EXPERIMENTAL PROCEDURES

Promoter DNA—The sequence of 160-bp promoter DNA (–110 to +50) used in this study was derived from λPR promoter sequence (supplemental data). It contained λPR sequence from –60 to +20 with –12G changed to T. Thus, both –10 and –35 hexamers were consensus. The rest of the sequence (–110 to –61 and +21 to +50) was unrelated random sequence DNA generated by a computer. Promoter DNA was assembled from two synthetic oligonucleotides: top strand 98-nt oligonucleotide (–110 to –13) and bottom strand 79-nt oligonucleotide (+50 to –29). The above oligonucleotides were mixed at 5 μM concentration in Klenow polymerase buffer and annealed (through the complementary 17-bp sequence at the 3′-ends of the oligonucleotides) by heating to 95 °C for 1 min and slowly (2 h) cooling down to room temperature. After annealing, dNTPs were added to 1 mM concentration, and Klenow fragment was added (5–8 units/μl) to fill in single-stranded regions of hybridized constructs. 160-bp promoter fragments containing mutations or mismatched base pairs were prepared as described above using top or bottom strand oligonucleotides with appropriate base substitutions. In the case of promoters with mismatched base pairs in the –10 region, 106 nt (–110 to –5) top oligonucleotides were used to prepare promoter fragments. The extended 25-bp complementary region in the case of this longer top oligonucleotide included the entire –10 region, allowing incorporation of mismatched base pairs into this region. Products of Klenow extension reactions were purified by ion exchange chromatography on a 1-ml Resource Q column (Amersham Biosciences). The column was eluted with a 30-ml salt gradient (0.45-0.90 mM NaCl in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA). The 160-bp promoter fragments were eluted from the column at ~0.65 mM NaCl. Pooled fractions were concentrated on Microcon Y-30 filter (Amicon). Concentration of promoter fragments was determined from absorbance at 260 nm. Some promoter templates for transcription assays were prepared by PCR amplification using promoters prepared as described above as a template.

Kinetics of Heparin-resistant Complex Formation—Kinetics of heparin-resistant complex formation was determined using an electrophoretic mobility shift assay (30). Experiments were performed at 25 °C in 20 mM Tris (pH 8.0) buffer containing 100 mM NaCl, 10 μM EDTA, 5 mM MgCl2, and 0.1 mg/ml bovine serum albumin. Reactions were initiated by mixing 4 μl of 18.75 mM DNA template with 1 μl of 1 μM holoenzyme. Final concentrations were 15 nM and 200 nM for the template and the holoenzyme, respectively. At this high saturating concentration of the polymerase, the observed rates of heparin-resistant complex formation were independent of polymerase concentration and thus reflected the kinetics of the steps following the initial binding of the polymerase to the promoter. Reactions were terminated at the desired time points by adding 2 μl of heparin–Ficoll mix. Final concentration of heparin was 0.2 mg/ml. Immediately after the addition of heparin, 7-μl samples of the reaction mixture were loaded on a 7.5% native polyacrylamide gel running at constant 80 V in TBE buffer. Gels were stained with Sybr Green and were imaged on Molecular Imager FX (Bio-Rad). Total fluorescence of free DNA bands was determined using the scanner software and plotted as a function of time. Pseudo-first order apparent rate constants were obtained from nonlinear fitting of the data to a single-exponential decay model. In order to measure kinetics of open complex dissociation, open complexes were first preformed in a 150-μl sample containing 15 nM template and 150 nM holoenzyme. After a 15-min incubation at room temperature, 3 μl of 10 mg/ml heparin were added (0.2 mg/ml final heparin concentration). At the desired time points, 5-μl samples were withdrawn and immediately loaded on a 7.5% native polyacrylamide gel running at constant 40 V in TBE buffer. The gels were stained and analyzed as described above.

Permanganate Footprinting—5′-Ends of promoter templates were labeled with 32P using T4 polynucleotide kinase for 30 min at 37 °C (5–10 pmol of 5′ termini in 30 μl of reaction mixture containing 70 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 5 mM dithiothreitol, 10 pmol of [γ-32P]ATP (6000 Ci/mmol), and 10 units of T4 polynucleotide kinase (New England Biolabs).
the QIAquick PCR purification kit (Qiagen). This labeling procedure produced DNA duplexes with both strands labeled at the 5'-end. Since the promoter sequence was positioned asymmetrically within promoter DNA fragments used, the footprints from both strands did not overlap each other when analyzed on polyacrylamide gel. Alternatively, DNA fragments with 32P label on one strand only were prepared by digesting promoter fragments with BglII or BamHI (for the lower strand labeling) and HindIII (for upper strand labeling). Following restriction enzyme digestion, dCTP, dGTP, and dTTP (0.2 mM final concentration) and 13 pmol of [α-32P]dATP (in the case of BglII and BamHI digest) or dATP, dGTP, and dTTP (0.2 mM final concentration) and 13 pmol of [α-32P]dCTP (in the case of HindIII digest) and 5 units of Klenow fragment were added. The reaction mixture was incubated at room temperature for 30 min. Radioactive labeled DNA fragments were purified with the QIAquick PCR purification kit (Qiagen). Oxidative modification with KMnO4 was performed with 5 nM labeled template, 100 nM holoenzyme in 10 mM transcription buffer. RNAP and template DNA were first mixed, and the open complex was allowed to form. The reaction was initiated by adding KMnO4 to a final concentration of 2 mM, and the mixture was incubated for 15 s at 37 °C. The reaction was quenched by adding 10 μl of 1% β-mercaptoethanol and 6 μg of calf thymus DNA in 50 μl of 10 mM Tris-HCl (pH 8.5). Reaction mixture was extracted with phenol, followed by chloroform extraction and ethanol precipitation. DNA pellets were dissolved in 100 μl of freshly prepared 1 M piperidine, and the samples were boiled for 10 min. Piperidine was extracted by chloroform, and DNA was precipitated by ethanol. Pellets were washed with 70% ethanol, 96% ethanol and were dried and dissolved in 7–10 μl of formamide colored with bromphenol blue. Samples were loaded on 8% sequencing gel. After the run, the gels were fixed in 5% acetic acid, dried on the gel dryer, and exposed with a phosphorus screen overnight. The images were scanned using PhosphorImager (Amersham Biosciences) and were analyzed quantitatively using ImageQuantMac version 1.2. KMnO4 footprints of naked DNA templates were performed in an analogous manner except that the reaction was initiated by adding KMnO4 to a final concentration of 2.5 mM followed by incubation for 3 min at 37 °C.

Transcription Assays—Transcription assays were performed in 50 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin at 37 °C for 30 min. Holoenzyme for transcription assays was reconstituted by mixing 2 μM core enzyme and 5 μM 70. Reconstituted holoenzyme was added to 10 μl of 10 nM template to a final concentration of 54 nM. Open complex was allowed to form for 10 min at 37 °C. Transcription was initiated by adding 2 μl of NTP mix (1.2 μl of 50 μM NTP, 0.56 μl of [α-32P]UTP (3000 Ci/mmol, 10 mCi/ml), 0.12 μl of 20 mM ATP, 0.12 μl of 10 mg/ml heparin). Reaction mixture was incubated for 10 min at 37 °C. The reactions were terminated by the addition of 10 μl of formamide-containing stop buffer. Transcription products were resolved by 20% denaturing urea PAGE. Bands of radioactive RNA products were visualized using PhosphorImager (Amersham Biosciences) and were quantified using the program ImageQuantMac version 1.2.

Stability of DNA Duplexes Containing Mismatched Bases—The relative stability of DNA duplexes containing various mismatched base pairs was determined using a simple partitioning assay (Fig. 5). 19-nt oligonucleotide (50 nM) corresponding to the fragment of promoter nontemplate strand around the −10 region (containing T at the −11-
position), labeled at the 5’-end with fluorescein, was mixed with a 39-nt oligonucleotide (100 nM) corresponding to the template strand sequence (containing A at the −11-position) and 79-nt oligonucleotide (500 nM) corresponding to the template strand sequence (containing various mismatched bases at the −11-position). Relative amounts of fluorescein-labeled 19-nt oligonucleotides hybridized to 39 and 79 oligonucleotides were measured by running the samples on a native polyacrylamide gel and scanning the gel using MolecularImager FX. The ratio of these two duplexes was used to calculate relative stability of the two duplexes according to a procedure analogous to that described in Ref. 22. Relative stability was calculated as a free energy difference between stability of 19-nt/39-nt duplex (containing a T-A base pair at position −11) and stability of 19-nt/79-nt duplex (containing the corresponding base pair mismatch at position −11). Stability measurements were made in 20 mM Tris-HCl (pH 8.0) buffer containing 100 mM NaCl, 10 μM EDTA, 5 mM MgCl₂, and 0.1 mg/ml bovine serum albumin. Essentially the same results were obtained if the samples were mixed at 25 °C or were first heated up to 95 °C followed by slow cooling to 25 °C.

RESULTS

Effects of Mutations in the −10 Region of the Promoter on the Kinetics of Promoter Melting and on the Binding Affinity of −10 Single-stranded Nontemplate Strand DNA Exhibit a Similar Pattern—In our previous studies, we have determined the energetic consequences of mutating each of the −10 promoter region consensus bases on the binding affinity of a model fork junction DNA construct containing −10 nontemplate strand in single-stranded form (22). These studies showed that mutations at positions −11 and −7 produced the largest decrease in the binding affinity.

In the present work, we assessed the role of sequence-specific interactions between RNAP and the nontemplate strand by determining if these interactions are involved in formation of kinetically significant intermediates in the promoter melting reaction. We prepared 13 mutant promoter constructs containing various single base pair substitutions in the −10 region. For each of these mutants, the kinetics of formation of heparin-resistant complex was examined. Resistance to the challenge with heparin is a hallmark of the formation of the first intermediate in the closed to open complex isomerization reaction (7). Fig. 1 illustrates results of these experiments. The formation of heparin-resistant complex was extremely fast with promoter containing the consensus −10 and −35 elements (Fig. 1, A and B). The reaction was essentially complete within the time required to mix the reagents (~15 s). In contrast, a promoter bearing an A to T substitution at position −11 exhibited much slower kinetics of heparin-resistant complex formation (Fig. 1, C and D). Although promoter melting was much slower in the case of this mutant, the final open complexes with the wild-type and mutant promoters had a similar structure, as judged by KMnO₄ probing experiments (Fig. 2). The same set of template strand thymine residues (with the obvious exception of the template strand −11T, which is missing in the mutant promoter) became susceptible to KMnO₄ oxidation in the presence of RNAP, indicating a qualitatively similar extent of promoter melting in the case of the wild-type and mutant promoter. Additionally, these probing experiments confirmed the slower kinetics of promoter melting in the case of the mutant promoter, since full footprint is established within 0.5 min in the case of the wild-type promoter, whereas only a very faint footprint is visible at this time point in the case of the mutant.

Fig. 3 shows the summary of the kinetics analysis of heparin-resistant complex formation with all 13 promoters. The value of the rate constant for the WT promoter should be considered as the lowest estimate (i.e. the rate is equal or higher than the value presented in Fig. 3), since the reaction was too fast to allow precise determination of the rate constant.
complex dissociation for all of the promoter mutants. Open complexes were allowed to form and were challenged with heparin. The time course of open complex dissociation was measured using native gel electrophoresis in a manner analogous to that used for open complex formation kinetics. Intensity of open complex bands remaining at each time point after the addition of heparin was measured, and the curves of residual intensity versus time were analyzed by nonlinear regression to determine apparent rate constants. The pattern of the effects of base mutations on the rate of open complex dissociation is shown in Fig. 4. The dissociation rates for mutants at position −11 were the highest (and ~2-fold higher compared with the WT promoter) with the exception of −11A to G mutations, for which the observed rate was lower than in the case of WT promoter. The rates for mutants at positions −12, −10, −9, and −8 were much lower (with the exception of the −12T to A). Interestingly, the rates observed for mutants at positions −10, −9, and −8 were lower than for the wild-type promoter. Comparison of the results illustrated in Figs. 3 and 4 indicated that mutations in the −10 region had a much more profound effects on the rate of promoter melting compared with the effects exerted on the rate of open complex dissociation.

Taken together, the analysis of the effects of base substitutions on the kinetics of open complex formation and dissociation showed that interactions involving the −11 adenine are involved in the formation of a kinetic intermediate on the pathway from closed to open complex formation.

**Nontemplate −11A Is Important for Initial Promoter Melting**—Data illustrated in Figs. 1–4 indicated an important role of −11A in the closed to open complex transition. This transition may involve at least two intermediates: conformational changes of RNAP-promoter complex (possibly including local melted DNA) or disturbance of −10 promoter element and melting of promoter DNA to produce a transcription bubble encompassing the transcription start site (1–3). We have thus performed an experiment to determine if adenine at position −11 of our model promoter constructs plays a role in initial melting of the promoter or in subsequent expansion of the transcription bubble. We have compared the effect of mutating the −11A in a context of a duplex promoter and in a promoter where this position was premelted by introducing a local mismatch at the −11 base pair. As was already illustrated in Figs. 1–4, an A/T to T/A mutation at position −11 (which preserves the duplex nature of the DNA) resulted in a dramatic decrease in the rate of heparin-resistant complex formation at all three temperatures tested (Fig. 5). Premelting −11A by introducing an A/A mismatch did not affect the rate of heparin-resistant complex formation in any notable manner. This is somewhat surprising and is in contrast with the previously reported enhancement of the rate of promoter melting resulting from introducing an A/A mismatch at position −11 (31). Our model promoter contained consensus −35 and −10 elements and was melted extremely fast (Fig. 1, A and B), which is probably a reason why we did not observe enhancement of melting upon introducing A/A mismatch into position −11. Importantly, substituting the nontemplate −11 A to T in a context of locally premelted template (by introducing a T/T mismatch) resulted in a relatively small (~2-fold) decrease in the rate of heparin-resistant complex formation relative to the rate observed for the A/A construct. This relatively small decrease can be contrasted with the ~16-fold rate decrease observed when the same nontemplate base was introduced in the −11 position in the fully double-stranded promoter construct. These data show that whereas the identity of the base at nontemplate −11-position is critical for efficient promoter melting, it becomes relatively unimportant once the promoter DNA has been locally premelted. Therefore, base-specific interactions of nontemplate −11 adenine seem to play a role only in the initial promoter melting (nucleation).

The interpretation of experiments shown in Fig. 5 can become very complicated if base mismatches used to obtain insights into the function of interactions of one specific base cause nonlocal changes in the DNA structure. For example, a base pair mismatch at position −11 is very likely to affect stability and/or conformation of the DNA duplex several base pairs away from the actual site of the mismatch (32). We have therefore designed a series of experiments to further probe the role of nontemplate −11 A in the nucleation of promoter melting (Figs. 6–8). These experiments involved introducing a series of defined structural perturbations to the −11 base pair. The use of perturbations with different capacity to induce nonlocal changes in the neighboring DNA duplex allows one to draw more definite conclusions from the results. An analogous approach has been very successfully applied in studies of base flipping facilitated by a DNA repair enzyme (33). In experiments illustrated in Figs. 6–8, we have used a mutant promoter construct containing a thymine at the nontemplate −11-position. This template is a very poor promoter compared with the WT promoter and exhibits an ~16-fold slower rate of heparin-resistant complex formation (Fig. 5).
Nucleation of Promoter Melting

We reasoned that if the deficiency of this mutant promoter was due to impaired promoter melting nucleation resulting from the absence of −11 adenine-specific interactions with the polymerase, perturbations of the −11 base pair that decrease the base pair stability without causing local melting of the DNA duplex should partially restore the promoter melting activity. This subtle destabilization would thus bypass the requirement of −11A-polymerase interactions for efficient nucleation and subsequent melting of the promoter.

A series of bases and base analogs were introduced at the template strand position −11 opposite nontemplate −11T (Fig. 6). Each of these substitutions should produce a different degree of local duplex destabilization. We initially used previously published values of destabilization free energy (ΔΔG) for these bases and base analogs paired with T (33), but we found poor correlation of some of these values with our experimental data. One obvious reason for such a discrepancy is that destabilization free energy for some of the base analogs could depend on the DNA sequence context. We have thus remeasured destabilization free energies in the context of DNA sequences used by us, using a simple partitioning assay illustrated in Fig. 6. In this assay, fluorophore-labeled oligonucleotide containing nontemplate strand promoter sequence (with a T at position −11) is mixed with a molar excess of two template strand oligonucleotides: one, shorter (serving as a reference), containing an A at position −11 and another, longer, containing a base analog at position −11. Oligonucleotides were used at concentrations that were much higher than dissociation constants of corresponding DNA duplexes, thus assuring that the binding reactions were under stochiometric conditions. Under these conditions, relative fractions of fluorophore-labeled oligonucleotide bound to the complementary oligonucleotides directly reflect the relative affinity constants, allowing straightforward calculations of destabilization free energies (22). The values obtained using these approaches (Fig. 6) were significantly different from the values reported previously in a different DNA sequence context (33).

For each of the T-base analog pairs incorporated into the −11-position of promoter DNA, we have measured the rate constants of heparin-resistant complex formation, and we plotted the logarithm of these rate constants as a function of destabilization free energies (Fig. 7A). The plot showed a remarkable linear correlation between these two parameters. Even very small perturbations to the base pair stability, which probably did not affect significantly the overall duplex nature of DNA template (e.g. due to nebularine or diaminopurine substitutions), produced a measurable increase in the rate of heparin-resistant complex formation. We also investigated the correlation between −11 base pair destabilization and transcriptional activity of the promoter (Fig. 7B). Whereas these data were noisier (due to a much more complex nature of the parameter measured), we observed a similar linear correlation between the logarithm of relative transcriptional activity of promoter variants and the destabilization free energies (Fig. 7B). This provided further confirmation of a direct link between −11 base pair stability and the efficiency of transcription initiation.

Overall integrity of DNA duplexes containing the base analogs used was demonstrated by their ability to produce sharp, stable bands in a native gel upon hybridization with the 19-nt nontemplate strand oligonucleotide (Fig. 6) (data not shown). To investigate the local properties of the DNA duplex in the vicinity of position −11, we probed the acces-

![FIGURE 6. Determination of DNA duplex destabilization due to various base pair mismatches introduced to position −11. A, design of the assay. A 19-nt fluorescein-labeled nontemplate strand DNA fragment containing T at −11 was mixed with a 2- and 5-fold molar excess of complementary DNA strand (39 nt) containing A at −11 and complementary DNA strand (79 nt) containing the indicated base analog at position −11. Relative fractions of labeled 19-nt DNA strand bound to 39- and 79-nt complementary strands are directly related the difference in DNA duplex stability between these two complexes. B, image (scanned using fluorescein settings) of the native polyacrylamide gel containing samples prepared as described in A. Values of ΔΔG were calculated as described under “Experimental Procedures.” D, diaminopurine; G, nebularine; dS, dSpacer.](image)

![FIGURE 7. Correlation between destabilization of DNA duplex (ΔΔG) due to a mismatch at position −11 and the rate of heparin-resistant complex formation (A) and transcription activity (B). Kinetics of heparin-resistant complex formation and transcriptional activity were measured using 160-bp DNA templates containing the following base analogs at the −11 template position paired with a T at the −11 nontemplate position: 1, A; 2, diaminopurine; 3, nebularine; 4, G; 5, T; 6, dSpacer.](image)
sibility of thymines in this region to oxidation by KMnO₄ in the naked DNA in the absence of the polymerase (Fig. 8). Accessibility of nontemplate −11T in templates where this base was paired with diaminopurine, nebularine, or G was similar to that observed in the T/A-containing DNA template, indicating that on average this base remained intrahelical. The increased accessibility of −11T to KMnO₄ oxidation became quite evident, with base analogs producing the highest degree of duplex destabilization (T and dSpacer; Fig. 8). Additional data are presented here indicating that interactions at (or in the near vicinity) of position −11 are specifically important in the initial stage (nucleation) of the promoter melting reaction. This adds another argument for the importance of RNA polymerase contacts with this position during promoter melting. Additionally, data presented here indicate that interactions at (or in the near vicinity) of position −11 are specifically important in the initial stage (nucleation) of promoter melting. This conclusion is derived from an observation that the identity of the non-template base at −11 becomes relatively unimportant when the −11 base pair is premelted and from observing a direct correlation between the rate of promoter melting and the stability of the base pair at position −11.

In summary, stability of the base pair at position −11 is directly correlated with the promoter melting activity of the polymerase. This correlation strongly supports the hypothesis that base-specific interactions between −11A and the polymerase facilitate promoter melting by stimulating initial melting nucleation at the upstream edge of the −10 region.

**DISCUSSION**

The mechanism of bacterial promoter DNA melting remains an unresolved problem. Recent results demonstrated that promoter melting activity of the polymerase is localized to a very small subset of polymerase-promoter interactions (19, 20). These interactions are limited essentially to contacts with the conserved −10 element. The ability of the σ²⁰ subunit to bind to one of the strands (non-template) of this element with high affinity and high sequence specificity (22) suggests an essential role of these interactions in promoter melting. Specific recognition of the adenine at the non-template strand −11-position is likely to be of special importance for promoter melting. A significant amount of experimental evidence has been accumulated in support of the important role of −11A. This is one of the most evolutionary conserved positions among bacterial promoters (21). It resides at the upstream boundary of the transcription bubble, where promoter melting is believed to be initiated (9). It is one of the two positions providing the largest energetic contribution to sequence-specific recognition of the non-template strand by the σ subunit (22). The N1 position of this adenine appeared to be essential for recognition by RNA polymerase, indicating the possibility of the existence of specific polymerase-promoter contacts that destabilize the −11 base pair (22). In a study of the effect of depurination of bases in the promoter on the rate of promoter melting, depurination at various positions resulted in stimulation of promoter melting presumably due to the reduction of helix stability (8). The single exception was depurination of the non-template strand −11A, emphasizing again a special role of this base in promoter melting.

In this study, we presented data that demonstrate that polymerase interactions with the base pair at position −11 play an important role in facilitating a kinetically relevant intermediate(s) in the promoter melting reaction. This adds another argument for the importance of RNA polymerase contacts with this position during promoter melting. Additionally, data presented here indicate that interactions at (or in the near vicinity) of position −11 are specifically important in the initial stage (nucleation) of promoter melting. This conclusion is derived from an observation that the identity of the non-template base at −11 becomes relatively unimportant when the −11 base pair is premelted and from observing a direct correlation between the rate of promoter melting and the stability of the base pair at position −11.

Our conclusions regarding the role of −11A are based on the results of studies involving introducing mutations into the −10 region of promoter DNA. This region is involved both in initial promoter binding by polymerase and in subsequent promoter melting. It is thus important to clarify why we can disregard the effects of the mutations on the initial binding step in our analyses. We could do this because in all experiments RNA polymerase was used at high saturating concentrations. At these concentrations, the observed rates of heparin-resistant complex
formation were independent of polymerase concentration and thus reflected the kinetics of the steps following the initial binding of the polymerase to the promoter.

Observation of a direct correlation between the relative stability of the −11 base pair and promoter melting activity or transcriptional activity (Fig. 7) is quite remarkable and provides the strongest support for the overall conclusions derived from this work. Whereas numerous studies have implicated a role of helix stability in promoter melting (3), as far as we know, the data illustrated in Fig. 7 provide the first evidence for such a direct link between local (possibly limited to a single base pair) duplex stability of the promoter and its function. These data provide a further indication that interactions of the polymerase with the consensus nontemplate strand −11A are directly involved in facilitating local destabilization of DNA duplex, since the deficiency in these interactions can be rescued by artificially induced local destabilization of the duplex.

Whereas it is tempting to assign these duplex destabilizing effects specifically to interactions with −11A, caution should be exercised in making such defined conclusions, since it is very difficult to limit DNA duplex perturbations, such as those used in this work, to just one base pair. However, since the enhancement in the rate of promoter melting can be observed even when the −11 base pair is destabilized by as little as ∼0.5 kcal/mol (Fig. 7A), which is not likely to produce significant effects in base pairs other than −11, interaction of RNAP with −11A remains the most probable candidate for the functionally important contact responsible for facilitating promoter melting nucleation. The exact mechanism by which this contact could facilitate promoter melting nucleation remains to be determined. One attractive possibility involves a promoter melting nucleation intermediate in which −11A would flip out of DNA helix. Such a role of the nontemplate −11A in the initiation of promoter melting has been suggested before in a form of the bind-nucleate-melt model proposed by Helmann and de Haseth (3). Extrahelical conformations of DNA bases are essential for the function of DNA repair enzymes and have been observed directly (34). The proposed role of −11A in the nucleation of DNA melting is mechanistically similar to base flipping by the DNA repair enzymes and is consistent with general biophysical characteristics of bubble formation within double-stranded DNA. Initial breaking of Watson-Crick hydrogen bonding and stacking interactions in DNA duplex is associated with high activation energy, whereas subsequent unzipping of additional base pairs adjacent to the initial unstacking reaction requires lower energy input (35). Recent studies on spontaneous DNA bubble formation using fluorescence correlation spectroscopy showed that bubbles of 2–10 base pairs with lifetimes in the 50-μs range can form at 37 °C in A/T-rich regions of low duplex stability. These studies also showed that DNA duplex remains zipped most of the time due to the high initiation energy for single base pair unzipping, but once a single base pair is unzipped the bubbles formed are not limited to a single base pair (35). It would thus be logical to expect that the critical rate-limiting step of promoter melting by RNAP could involve a base-flipping event. Once such an intermediate is formed, further unzipping of DNA duplex should proceed relatively rapidly. Adenine at position −11 would be the most logical candidate for such a base-flipping event. One could envision that RNAP could either actively promote the flipping of −11A or passively take advantage of spontaneous dynamics of this base and use the −11A-specific interactions to stabilize extrahelical conformation of the base. Direct experimental evidence for an intermediate in the promoter melting reaction involving a base-flipping event will be very difficult to obtain due to the likely transient nature of such an intermediate but will be essential for the definite confirmation of the proposed role of −11A.

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