Mechanistic Differences in Promoter DNA Melting by Thermus aquaticus and Escherichia coli RNA Polymerases

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Formation of strand-separated, functional complexes at promoters was compared for RNA polymerases from the mesophile Escherichia coli and the thermophile Thermus aquaticus. The RNA polymerases contained sigma factors that were wild type or bearing homologous alanine substitutions for two aromatic acids involved in DNA melting. Substitutions in the σ^A subunit of T. aquaticus RNA polymerase impair promoter DNA melting equally at temperatures from 25 to 75 °C. However, homologous substitutions in σ^B render E. coli RNA polymerase progressively more melting-defective as the temperature is reduced below 37 °C. The effects of the mutations on the mechanism of promoter DNA melting were investigated by studying the interaction of wild type and mutant RNA polymerases with “partial promoters” mimicking promoter DNA where the nucleation of DNA melting had taken place. Because T. aquaticus and E. coli RNA polymerases bound these templates similarly, it was concluded that the different effects of the mutations on the two polymerases are exerted at a step preceding nucleation of DNA melting. A model is presented for how this mechanistic difference between the two RNA polymerase could explain our observations.

Transcription in bacteria is catalyzed by DNA-dependent RNA polymerase (RNAP), a key enzyme for gene expression and its regulation (1, 2). Specific recognition of promoter DNA is mediated by an initiation subunit (sigma factor). The functional RNAP “holoenzyme” results when the sigma factor binds to the catalytic component, the “core” enzyme. A highly conserved region of 18 amino acids (designated region 2.3) of the main bacterial sigma factors has been shown to play a role in the melting process (3–6). Aromatic amino acid side chains in this region are positioned on the same side of an α helix, poised to interact with the exposed bases of the promoter DNA (7, 8). Studies with Escherichia coli (Eco) RNAP have indicated that upon RNAP binding to promoter DNA, an unstable closed complex (RPc) is formed, followed by at least two additional intermediates (I₁ and I₂) before the initiation-competent, stable open complex (RPo) is formed (9, 10), in which a 14-bp region of the promoter DNA, including the start site of transcription (2), has been melted.

R + P ↔ RPc ↔ I₁ ↔ I₂ ↔ RPo

SCHEME 1

In addition to the promoter DNA, the RNAP is thought to undergo conformational changes as well (9, 10).

The past 6 years have seen a dramatic increase in structural information for bacterial RNAP, primarily because of the successful crystallization of the RNAP from two thermophilic bacteria, Thermus aquaticus (Taq) and Thermus thermophilus (8, 11–14). The RNAP from these two organisms share extensive homology with all subunits of Eco RNAP, including the primary sigma factors. Four amino acids are different between the DNA melting regions (2.3) of Taq σ^A and Eco σ^B, but in three of the four cases the differences involve amino acids with chemically similar side chains. It is not well understood to what extent the mechanism of open complex formation at elevated temperatures for a thermophilic RNAP, such as the Taq enzyme, might differ from that for Eco RNAP to take advantage of the temperature-induced instability of the DNA helix (15–18).

In this work we have compared Taq and Eco RNAP-containing sigma factors that were both wt or bearing homologous amino acid substitutions in region 2.3, with respect to their ability to interact with promoters and with model templates designed to emulate the state of promoter DNA in various intermediates of scheme 1. Our studies revealed clear differences in the mechanisms of promoter DNA melting by Taq and Eco RNAP. Although the Taq σ^A mutants were equally defective at high and low temperatures, the defects caused by substitutions in Eco σ^B were mitigated at higher temperatures (this paper and Refs. 3, 5, 6, and 19). We provide evidence that temperature and the mutations affect the I₁ to I₂ step in Scheme 1 for both RNAP. The difference would lie in the RPc to I₁ conversion, which would be affected by both temperature and the mutations for Eco RNAP but only one or the other for Taq RNAP.

EXPERIMENTAL PROCEDURES

Materials—Oligodeoxynucleotides were synthesized by Invitrogen. [γ-<sup>32</sup>PP]ATP was purchased from PerkinElmer Life Sciences, DNA-modifying enzymes from either New England Biolabs or Roche Applied Science, and Eco RNAP core from EpiCenter. All of the chemicals were from Sigma or Fisher.

Protein Purification—A recombinant Taq σ^A gene (rp0D) in pET28a, preceded by the T7 promoter and with an N-terminal hexahistidine tag (a gift from the laboratory of K. Severinov), was used for overexpression and purification of σ^A by Ni<sup>2+</sup> affinity chromatography. The pET28TaABCZ plasmid (also a gift from K. Severinov) harboring the genes for the α, β, β’ (with C-terminal hexahistidine tag), and ω subunits of core RNAP was used for expression and purification of core RNAP by Ni<sup>2+</sup> affinity and Resource Q (Amersham Biosciences) anion

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The abbreviations used are: RNAP, RNA polymerase; Eco, E. coli; Taq, T. aquaticus; T, thymine; SF, short fork; LF, long fork; EMSA, electrophoretic mobility shift assay; SS, single-stranded; wt, wild type.
exchange chromatography (16, 17). Eco δ70 was purified as described
(5).

Reconstitution Reactions—Purified RNAP core and σ factors were incubated at a molar ratio of 1:5 for 1 h on ice to obtain RNAP holo-
enzyme at a concentration of 400 nM (expressed as the concentration of the limiting core component). Mutagenesis of Taq σr was carried out with the QuiChange kit from Stratagene per the manufacturer's instructions.

DNA Labeling and Annealing—DNA oligonucleotides, purified as described, were 5' end-labeled with 32P by polynucleotide kinase in a reaction containing [γ-32P]ATP (20). Unincorporated [γ-32P]ATP was removed using Bio-Rad Micro Bio-Spin 6 chromatography columns. Annealing of complementary DNA strands was performed in a 40-μl reaction volume containing 25 mM Tris-HCl, pH 7.9, 50 mM NaCl, 100 mM 32P-labeled DNA, and 150 mM unlabeled complementary strand. The reactions were incubated at 90–95 °C in a heat block for 5 min, followed by slow cooling to room temperature. The concentration of the annealed DNA is expressed as the concentration of the limiting, radiolabeled strand.

Electrophoretic Mobility Shift Assay—Each reaction (10 μl) contained, in Tris binding buffer (40 mM Tris-HCl, pH 8 (adjusted at the temperature of the reaction), 40 mM KCl, 10 mM MgCl2), 10 nM annealed DNA, and 65 nM of RNAP holoenzyme. If necessary, quantitative binding analysis was observed with 31P-ATP (20). Unincorporated [γ-32P]ATP was removed using Bio-Rad Micro Bio-Spin 6 chromatography columns. The reactions were stopped by placement on dry ice. Ten μl of formamide dye (10 mM NaOH, 1 mM EDTA, 80% formamide, 0.1% xylene cyanol FF, 0.1% bromphenol blue), and 500 μl of mineral oil was layered over the solution to prevent evapora-
tion. To assay for formation of stable complexes, the reactions were challenged with 100 μg/ml of heparin and incubated for an additional 10 min. For loading, 2 μl of dye solution (45% glycerol, 50 mM sucrose, 0.1% bromphenol blue, and 0.1% xylene cyanol FF) was added to each reaction, and 9 μl was applied to a running 4% native gel poured and run in TAE buffer (40 mM Tris acetate and 1 mM EDTA). The gels were run in TAE buffer (40 mM Tris acetate and 1 mM EDTA). The gels were run at low voltage (90–100 V) for 1–2 h in a thermostatted Protein II xi cell electrophoresis unit (Bio-Rad). After drying, the gel was analyzed by phosphorimaging (Molecular Dynamics) using ImageQuant 5.2 soft-
ware. The percentage of the (radiolabeled) DNA present in RNAP-DNA complexes.

KMMeO Probing—Annealed DNA (30 nM) was preincubated in Tris binding buffer at the desired temperature for 1 min. The reactions (final volume, 20 μl) were started by addition of prewarmed RNAP to 100 nM and incubated for 1 min at the same temperature. Heparin was added to a final concentration of 100 μg/ml, and incubation was continued for an additional 2 min. Then 1 μl of a freshly made 21 mM stock of KMMeO4 was added for a final concentration of 1 mM. After 30 s, 5 μl of stop solution containing 1.5 M NaOAc, pH 8, 4 mg/ml glycogen, and 300 mM β-mercaptoethanol was added, and the reactions were placed on ice. After ethanol precipitation, the dried pellets were redissolved in 70 μl of 1 mM piperidine, and the solutions were incubated for 20 min at 90 °C. The RNAP was reprecipitated by placing the samples in dry ice. Ten μl of the reprecipitated RNAP was added to the thawing reactions, and the DNA was ethanol-precipi-
tated again. The dried pellets were taken up in distilled H2O, dried down, dissolved in 12 μl of formamide dye (10 mM NaOH, 1 mM EDTA, 80% formamide, 0.1% xylene cyanol FF, 0.1% bromphenol blue), and loaded onto a sequencing gel, poured, and run in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). After electrophoresis, the gel was dried, and the bands were revealed by phosphorimaging (Molecular Dynamic-
ses). Analysis of the images was performed using ImageQuant 5.2 software. To determine the RNAP-induced fractional melting of a promo-
der Duplex, the fraction for RNAP + Duplex DNA (obtained by dividing the radioactivity in the −7 to +2 region by the sum of this radioactivity and that of the radioactivity of full-length Duplex DNA) was corrected for the intrinsic reactivity of thymine (T) residues at each temperature, as described under “Results.” In the plots of Fig. 2, all of the values of the fractional melting were normalized to values obtained at 65 °C for Taq RNAP and 45 °C for Eco RNAP (the highest KMMeO4 reac-
tivities in the −7 to +2 region).

RESULTS

Template Design—To compare the temperature dependence for open complex formation by the Taq and Eco RNAP under the conditions employed in our experiments, the same Duplex promoter DNA template (Fig. 1) was used, taking advantage of the fact that the primary sigma factors of the two RNAP have similar promoter specificities. The sequences of all templates were derived from that of the Duplex DNA, which

is a sequence variant of the Pm promoter of bacteriophage λ, modified to contain consensus −10 and −35 regions. Additionally, G-C bp were introduced at the downstream end of the DNA to prevent fraying of the Duplex and at −13 to prevent fraying of the fork junction templates, short fork (SF) and long fork (LF). Taq RNAP was found to poorly form heparin-resist-
tant, open complexes with two other promoters tested on short (<100 bp) DNA fragments (data not shown): the Pα promoter of bacteriophage λ and a T. thermophilus promoter (21). Another promoter with consensus −10 and −35 regions also bound well to Taq RNAP (data not shown). The above promoters were all used efficiently by Eco RNAP. Our results also suggest that the Duplex sequence is a better promoter for Eco RNAP at 37 °C than for Taq RNAP at 65 °C (Fig. 2).

Stable Promoter DNA Complexes with wt and Mutant RNAP: Electrophoretic Mobility Shift Assay—The formation of stable RNAP-promoter complexes with Taq and Eco RNAP was com-
pared using the electrophoretic mobility shift assay (EMSA), following a heparin challenge of the binding reactions. The RNAP contained wt sigma factors or those bearing alanine substitutions for two aromatic amino acids in region 2.3 known to be important for promoter melting by Eco δ70 (Tyr430 and Trp433) or the homologous residues (Tyr253 and Trp256, respective-
ly), for Taq σA. The reactions were carried out at “high” (37 °C for Eco; 65 °C for Taq RNAP) and “low” temperatures (9 °C for Eco; primarily 37 °C for Taq RNAP). The incubation time between RNAP and promoter DNA was 30 min for the EMSA experiment shown in Fig. 2. In all cases the gel analysis was carried out at the temperature of the incubation.

A typical gel is shown in Fig. 2, which displays the interac-
tion of RNAP with Duplex promoter DNA at 37 °C. This was a low temperature for Taq RNAP, which was added to the reactions shown in Fig. 2A. In Fig. 2B, Eco RNAP was used, with 37 °C now being a high temperature. For Taq RNAP at 37 °C, a fainter complex band is seen below the main one. Higher temperatures cause the intensity of this band to decrease, rendering it invisible at 65 °C. We have verified that this band is not due to DNA binding by a complex of Taq core and any contaminating Eco δ70. This band may be due to the L2 complex, but we have not further addressed this issue.

The results of binding data carried out at several tempera-
tures are shown in Fig. 2 (C and D). For Taq RNAP a steep drop in extent of binding is seen between 37 and 33 °C (Fig. 2C); with Eco RNAP a smaller decrease is seen (Fig. 2D) between the high and the low temperatures tested. Interestingly homol-
ogous substitutions in δ70 and σA displayed different behavior. Both the Y253A and W256A single substitutions and the dou-
ble substitution adversely affected the ability of Taq holo-
enzyme to form stable complexes at the promoter at all tempera-
tures tested, with the extent of complex formation decreasing in the order wt > Y256A or Y253A > Y253A/W256A. However, with the Eco RNAP, only the Y430A substitution reduced the ability of the Eco holoenzyme to form a stable complex and then much less at 37 °C than 9 °C. This cold sensitivity is similar to that previously observed at other Eco promoters (5, 6). Neither the W433A substitution nor the Y430A/W433A double substi-
tution negatively affected the binding of Eco holoenzyme to promoter DNA at either temperature, in contrast to the results from our previous kinetic studies (6). It also appears that the Trp433 substitution actually can compensate for the defect of the Y430A substitution. We do not have an explanation for this finding, but in contrast to the templates used in our previous work (5, 6), a consensus promoter was used here (Fig. 1, Du-
pex), as required for Taq RNAP. For this promoter, the kinetics of open complex formation, except with Eco RNAP at 9 °C, are too fast to be measured by manual mixing methods. In addition
elements as well as the moter DNA labeled with $^{33}$P. Several controls are shown in Fig. 3.

The temperature optima of Taq and Eco RNAP (see below) and opened by RNAP (Fig. 1). Results from experiments carried out at base noncomplementarity in the region where promoter DNA is exposed T residues at each temperature. This was accomplished parallel reactions were carried out to determine the reactivity of T residues in the region of noncomplementarity of the Mismatch template is temperature-dependent; the bands are more intense at 65 °C than at 45 °C (Fig. 3A, compare lanes 5 and 11).

In the presence of RNAP, the reactivity of most T residues in the mismatched region increased significantly (compare lanes 4 and 5 and lanes 10 and 11). The patterns of T reactivities for double-stranded promoter DNA in the presence of RNAP are shown in lanes 3 and 9. The reactivities of the −10 and −7 T's on the Duplex template are reduced in the presence of either RNAP but on the Mismatch template by Taq RNAP only. Other differences between the two RNAP are the enhanced oxidation and the reduced oxidation of the −6 T seen with Eco RNAP. The different patterns likely reflect subtle differences in the way the two RNAP interact with Duplex DNA, resulting in different extents of protection for the less oxidized Ts or of exposure for the more oxidized Ts.

Temperature Dependence of Strand Opening by wt Taq and Eco RNAP—Experiments with Duplex DNA were carried out over a range of temperatures with both RNAP. The results were first normalized at each temperature to the intensities of the bands for the Ts at positions −7, −6, −1, and +2 for the complex of each RNAP and Mismatch DNA, to account for the intrinsic temperature dependence of the oxidation reaction. Next these values were normalized to the temperature optima for each polymerase (45 °C for Eco and 65 °C for Taq RNAP; Fig. 3B). Broad optimal temperature ranges are seen for both Eco and Tag RNAP, clearly showing the greater resistance of the Taq RNAP to thermal inactivation. With Tag RNAP, strand separation is two-thirds of maximal at 37 °C, but interestingly for the RNAP of the closely related bacterium T. thermophilus,

the strong Duplex promoter may inherently be more refractory to the deleterious effects of some of the mutations.

Determination of Strand Opening by $^{18}$O$_2$ Probing—Correction for the Intrinsic Temperature Dependence of the Oxidation Reaction—The above experiments demonstrated cold sensitivity of the Eco $\sigma^{70}$ Y430A mutant (Fig. 2D), but no such cold sensitivity was seen with any of the mutant Taq $\sigma^{5}$ tested, indicating that higher temperatures could not compensate for the melting defect of these mutations. In view of the possibility that the EMSA experiments (above) did not reflect open complex formation, the results were verified by using a direct assay for strand separation, monitoring the RNAP-dependent increased sensitivity of T residues to oxidation by $\text{KMnO}_4$ (22, 23).

First, the temperature dependence of the process was determined for both Taq and Eco RNAP. To correct for the temperature dependence of the intrinsic reaction rate of the oxidation, parallel reactions were carried out to determine the reactivity of exposed T residues at each temperature. This was accomplished with the Mismatch template, partially strand-separated because of base noncomplementarity in the region where promoter DNA is open by RNAP (Fig. 1). Results from experiments carried out at the temperature optima of Taq and Eco RNAP (see below) and including several controls are shown in Fig. 3A. RNAP and promoter DNA labeled with $^{35}$S at the 5' end of the nontemplate strand were incubated together for 1 min prior to a 2-min heparin challenge (to remove any end-bound RNAP) and exposure to $\text{KMnO}_4$. At each temperature, the appearance of bands caused by DNA cleavage was shown to be dependent on the presence of $\text{KMnO}_4$. In the absence of RNAP, the reactivity of T residues within the region of noncomplementarity of the Mismatch template is
which has a somewhat higher optimal growth temperature, at 37 °C no open complex formation was observed at the λ PR promoter (18).

As had long been known for the Eco RNAP (24) and was demonstrated here (Fig. 3B) and elsewhere (16, 18, 25) for thermophilic RNAP as well, promoter strand opening by wt RNAP displays a steep decrease at low temperatures. It was not possible to observe the high temperature inactivation of Taq RNAP, because of significant melting of the free double-stranded promoter DNA at temperatures of 80 °C and above (data not shown). Even so, the range of temperatures over which the extent of strand separation is within at least 60% of maximal is broader by greater than 10 degrees for the Taq than for the Eco RNAP. Only for Eco RNAP at 9 °C, significant additional melting (2–4-fold) was observed upon further incubation of RNAP and promoter DNA beyond the 1 min used in this experiment (data not shown). Thus the steep drop off at low temperatures in the case of Eco RNAP may in part be due to the slow kinetics of open complex formation under these conditions.

**Strand Opening by Mutant Taq RNAP**—The results for the interaction of the wt and mutant Taq RNAP with the Duplex promoter DNA at 37 °C are shown in Fig. 4A. Again, the extent of strand opening was calculated by comparison of complexes of Taq RNAP with Duplex and Mismatch DNA. The 65 °C lanes (lanes 10 and 11) are shown for normalization purposes. The percentage bound was calculated for each lane and plotted at various temperatures. The interaction of the wt and mutant Taq RNAP with Duplex and Mismatch DNA. The 65 °C lanes (lanes 10 and 11) are shown for normalization purposes. The percentage bound was calculated for each lane and plotted at various temperatures.

**Promoter DNA Melting by T. aquaticus RNA Polymerase**

**FIG. 2.** Taq and Eco RNAP binding to Duplex promoter DNA at various temperatures, as determined by EMSA. 10 nM Duplex DNA was incubated with 65 nM RNAP for 30 min prior to a heparin challenge for 10 additional minutes. The reactions were then loaded onto a running native gel to separate RNAP-DNA complexes (bound) from free DNA, as indicated in the figure. A and B, typical EMSA gels for Taq and Eco RNAP, respectively, binding to Duplex DNA at 37 °C. Only the regions of the gel containing the bound and free DNA are shown. C and D, summary of binding experiments at different temperatures for Taq and Eco RNAP, respectively. All of the numbers are the averages of at least two determinations. YW contains both the Y to A and W to A substitutions in either Taq or Eco RNAP, respectively, binding to Duplex DNA at 37 °C. These results indeed reflected formation of open complexes. These error bars indicate the spread between two independent experiments.

The Interaction of Taq and Eco RNAP with Fork Junction Templates Is Similarly Affected by Mutations in Region 2.3 of Sigma—To compare the Taq and Eco RNAP at a mechanistic level, formation of stable RNAP-DNA complexes with the mutants with Duplex DNA. The results of experiments for Taq RNAP at 37 and 65 °C are shown in Fig. 4B. Within experimental error, the patterns are similar to the results of the binding studies (Fig. 2C), again confirming that the EMSA results indeed reflected formation of open complexes. These experiments (Fig. 4) provide further evidence that high temperatures (up to 75 °C; KMnO₄ probing; data not shown) do not compensate for the melting defects of the mutant Taq RNAP.
model DNA templates shown in Fig. 1 was investigated. These are partial promoters, designed to mimic the conformation of DNA for intermediates in the process of open complex formation (20, 26). Nucleation of promoter DNA melting (the I1 to I2 step) likely involves forcing the $\text{Tt}^\text{11A}$ out of the DNA helix and into a pocket on the RNAP (“base flipping”) with concomitant acquisition of stability to heparin challenge (10, 27). The fork junction templates (Fig. 1, Short Fork and Long Fork) are completely double-stranded except for a single-stranded overhang including the base corresponding to the $\text{Tt}^\text{11A}$. Their use allows study of $\text{Tt}^\text{11A}$ capture while bypassing major conformational changes in RNAP (see “Discussion”), necessary for facilitation of open complex formation at promoters (10). We believe that a heparin-stable complex between RNAP and a fork junction DNA template mimics I2 (scheme 1), also heparin-resistant, rather than an earlier intermediate in open complex formation (20, 28).

Formation of a heparin-resistant complex between the SF template and either Taq or Eco RNAP, as determined by EMSA, was much better at the low rather than the high temperatures for each RNAP (Fig. 5, A and B). For wt Taq RNAP no heparin-resistant binding was observed at 65 °C but increasingly greater extents at 37 °C and lower temperatures, whereas wt Eco RNAP bound about 40% of the SF DNA at 9 °C and little at 37 °C. The lack of binding of SP to Taq RNAP at 65 °C was not due to melting of its double-stranded DNA region. SF DNA and LF DNA have identical double-stranded DNA regions, and from the high extents of binding of LF to wt Taq RNAP at 65 °C (Fig. 5C) it is concluded that its two strands are annealed, because single-stranded (SS) DNA would not bind (Fig. 6A). The mutant Taq holoenzymes did not display detectable binding to SF DNA at 37 °C. At lower temperatures the W256A substitution is seen to be progressively less deleterious, but the Y253A and double mutant are greatly impaired in SF DNA binding at all of the temperatures tested. For Eco RNAP, the mutants showed a similar pattern of defects in binding to the SF template. Particularly, the Y430A and YW mutants bound weakly even at the low temperature, whereas binding was only reduced by about 30% for the W433A substitution. The homologous Y253A (Taq) and Y430A (Eco) substi-
tutions greatly affect SF binding, indicating that this tyrosine residue is important for carrying out the $I_1$ to $I_2$ isomerization in Scheme 1.

LF DNA (Fig. 1) is an extension of the SF DNA, with an overhang that includes the exact —10 element, representing a later phase in the melting process. As previously observed with Eco RNAP (28, 29), Taq RNAP also bound better to LF than to SF DNA. wt and mutant Taq RNAP binding to LF DNA was ~90% at 37 °C (Fig. 5C). The lack of an effect of the substitutions is not due to saturation of the binding reaction, because a similar pattern is observed when the maximal extent of binding to wt RNAP is 65–70% at 9 °C and 60–65% at 37 °C (data not shown) for reactions containing lower concentrations of protein and DNA. The LF DNA allowed determination of the effects of the mutants on fork junction template binding at higher temperatures. At 65 °C, wt Taq RNAP still bound over 50% of the LF DNA, but the aromatic mutants were clearly defective (Y253A more so than W256A), stressing the importance of the wt residues for binding at this temperature. For Eco RNAP a similar pattern was observed; LF DNA was bound equally well by wt and mutant RNAP at a low temperature (9 °C), but at the higher temperature (37 °C) the mutants exhibited large defects (Fig. 5C).

**Fig. 6. Taq and Eco RNAP binding to single-stranded and mismatched DNAs at various temperatures.** The boxes contain keys to the color coding of the bars. The mutants are as described in the legend to Fig. 5. All of the reactions were analyzed on nondenaturing gels. The reactions labeled high contained 65 nM RNAP and 10 nM DNA (as in most of our experiments), those labeled low contained 10 nM RNAP and 2 nM DNA. The absence of a bar graph indicates a lack of detectable binding. All of the numbers are the averages of at least two determinations. A, interaction of Taq RNAP with SS DNA. The reactions and quantification were as described in the legend to Fig. 5, except for the lack of a heparin challenge. B, interaction of Eco and Taq RNAP with Mismatch DNA. The experiments with Eco RNAP were done at high concentrations, and those with Taq RNAP at low concentrations (see above). The reactions and quantification were as described in the legend to Fig. 5.

The Interaction of RNAP with Single-stranded Templates Is Temperature-sensitive—To compare the role of aromatic residues in the establishment and maintenance of DNA strand separation in an open RNAP-promoter complex, the ability of the mutants to interact with SS DNA (30–33) (Fig. 1) was determined. Heparin was not added to these reactions, because complexes of Eco RNAP and single-stranded DNA do not survive such a challenge. Surprisingly, a significant fraction of SS DNA-Taq RNAP complexes were observed to persist after a heparin challenge. No binding of SS DNA to wt or mutant Taq RNAP was observed at 65 °C, but at 37 °C, about 90% of the SS DNA bound to wt and the W256A Taq RNAP mutant. Complex formation with the Y253A and the YW mutant Taq RNAP was considerably less (Fig. 6A). Experiments with reduced concentrations of the reactants revealed a large increase in the affinity of RNAP for SS DNA as the temperature was further reduced from 37 to 9 °C (Fig. 6A). Eco RNAP has a lower affinity than Taq RNAP for the SS DNA; with Eco wt RNAP at 9 °C, just 4% binding of SS DNA was observed, but 20% was observed with the W433A mutant (data not shown). However, no detectable binding was observed to Eco RNAP at 37 °C (data not shown) for the same SS DNA that showed 90% binding to Taq RNAP. Thus the effect of temperature on the SS DNA-RNAP interaction cannot be exerted solely through the structure of the SS DNA. A similar conclusion with respect to promoter DNA was reached in comparisons of DNA binding by Eco RNAP and the RNAP from the thermophile *Thermotoga maritima* (15).

Only one amino acid residue in region 2.3 of each sigma factor is clearly different between Eco $\sigma^70$ and Taq $\sigma^A$: Eco Gly$^{424}$ versus Taq Arg$^{247}$. We investigated the potential functional significance of this difference because thermophilic proteins are generally found to have more charged residues (34) than their mesophilic counterparts. Surprisingly, the R247G substitution in $\sigma^A$ did not affect promoter DNA binding at any temperature tested. The only observed effect of the R247G substitution was a small (1.5-fold) but significant enhancement at 37 °C of the binding of SS DNA to the mutant Taq RNAP (data not shown).

Interaction of Taq RNAP with a Mismatched Template—To determine whether the mutated RNAP were defective beyond their ability to cause DNA strand separation, we determined their ability to bind Mismatch DNA (Fig. 1). Because little or no disruption of base pairing would be required for formation of a strand-separated complex analogous to an open complex, use of this template was expected to obscure promoter melting defects (19). In view of the high affinity of Taq RNAP for Mismatch DNA, lower concentrations were used to avoid saturating conditions. The interaction is largely unaffected by temperature. This may be due to cancellation of a temperature sensitivity such as that observed for binding of single-stranded and forked DNAs and the temperature dependence for open complex formation at promoters. Within experimental error the effects of the alanine substitutions were mitigated at all three temperatures tested (Fig. 6B), in contrast to the results for the Duplex DNA where the substitutions greatly impeded open complex formation (Fig. 2C). These results show that the major roles of the Tyr$^{253}$ and Trp$^{256}$ residues of Taq $\sigma^A$ are DNA melting rather than binding. Studies from Gralla’s group as well as our own group led to similar conclusions for the Tyr$^{430}$ and Trp$^{433}$ residues of Eco RNAP (4, 6, 28, 35). It is unclear why the results obtained here with the Mismatch and Duplex DNAs were not as readily interpretable: The defect of the Y430A substitution was similar for both templates, whereas the Trp$^{433}$ and YW substitutions did not affect the binding to either template (Figs. 6B and 2D).
DISCUSSION

Comparison of the Effects of Temperature and of Substitutions in Region 2.3 of Sigma—Alanine substitutions for Tyr<sup>253</sup> and Trp<sup>256</sup> of Taq σ<sup>α</sup> are deleterious to promoter DNA melting over a wide range of temperatures (Figs. 2C and 4B) up to 75 °C (data not shown). On the other hand, with Eco RNAP, the homologous substitutions have negligible effects at 37 °C, whereas at 9 °C the substitution at Tyr<sup>430</sup> leads to a 2-fold reduction in stable complex formation (Fig. 2D). For open complex formation by Eco RNAP at a weaker promoter, a more striking cold sensitivity was observed (5, 6). For Bacillus subtilis RNAP, the homologous mutants have been found to be cold-sensitive as well (3, 19). The lack of cold sensitivity for the Taq RNAP mutants contradicted our hypothesis that for Taq RNAP, active at higher temperatures, the effects of alanine substitutions in the DNA melting region of the sigma factor would be more readily compensated. Assuming similar roles for homologous amino acids, our results indicate that the mechanisms of open complex formation are different for Eco and Taq RNAP.

To better understand the differences in promoter melting by Taq and Eco RNAP, we used fork junction templates to determine whether the mutants affected the conversion of I<sub>1</sub> to I<sub>2</sub>, the first stable intermediate in the formation of an open promoter complex. Our rationale for their use is that they lack downstream DNA, which may be necessary for RNAP jaw contacts that drive conformational changes in RNAP and DNA bending (10). Therefore, the RP<sub>c</sub> to I<sub>1</sub> step is bypassed through use of fork junction templates, which report solely on the nucleation step of the melting process. For Taq and Eco RNAP, mutation of the homologous Tyr and Trp residues at positions 253 and 430 and positions 256 and 433 in region 2.3 of σ<sup>α</sup> and σ<sup>70</sup>, respectively, was shown to similarly affect formation of a heparin-stable complex with the fork junction templates. This is consistent with our previous finding that the mutations do not affect formation of the initial heparin-sensitive complex between Eco RNAP and fork junction DNA and with the absence of other observable intermediates prior to formation of the first heparin-resistant complex (28). Taq and Eco RNAP also behave similarly in binding to fork junction DNA templates in that binding becomes increasingly better at lower temperatures. We would therefore conclude that temperature and region 2.3 mutations have similar effects on the conversion of I<sub>1</sub> to I<sub>2</sub> for Taq and Eco RNAP.

In terms of the mechanism shown in Scheme 1, for Eco RNAP the step converting RP<sub>c</sub> to I<sub>1</sub> is affected by the substitutions as well, based on previous data. First, Eco RNAP bearing multiple substitutions of aromatic amino acid residues in region 2.3 formed promoter complexes with properties characteristic of the RP<sub>c</sub> complex, i.e. a short DNase I footprint, lack of strand separation, and heparin sensitivity/low stability (5). Second, these same RNAP were able to be recruited by an activator of transcription that stimulates formation of the RP<sub>c</sub> complex (36). Thus the RP<sub>c</sub> to I<sub>1</sub> and I<sub>1</sub> to I<sub>2</sub> conversions for Eco RNAP are both affected by the region 2.3 mutations in σ<sup>70</sup>, and as shown by Record and co-workers (10), also by temperature.

For Taq RNAP it is unknown which steps on the path to open complex formation are temperature-dependent, or even whether open complex formation proceeds through intermediates similar to those in Scheme 1. However, in view of the different effects of the base substitutions in region 2.3, it is likely that the mechanisms are different for Taq and Eco RNAP. A simple but, based on available data, not unique explanation for the independent effects of the substitutions and temperature for Taq RNAP would be that the step from RP<sub>c</sub> to the next intermediate would not involve the same RNAP conformational changes thought to occur at the RP<sub>c</sub> to I<sub>1</sub> step for Eco RNAP (10). Then the accompanying distortion of promoter DNA would not be available to facilitate nucleation and suppress the effects of the mutations in Taq σ<sup>α</sup>. However, this could still occur at a subsequent step.

The Role of Single-Stranded DNA in Stable RNAP-Promoter Complex Formation—For Taq RNAP, the Mismatch DNA template was able to rescue the deleterious effects of alanine substitutions for Tyr<sup>253</sup> and Trp<sup>256</sup> on complex formation with promoter DNA (Fig. 6B) at all three temperatures investigated. Similar results had previously been obtained for RNAP containing the homologous substitutions in B. subtilis σ<sup>α</sup> (19). Apparently, if the DNA is already melted, the substituted aromatic groups are less important to formation of a stable complex. However, KMnO<sub>4</sub> probing experiments still provided evidence for interaction of both Taq and Eco RNAP with the single-stranded DNA region of the Mismatch DNA (Fig. 3A). The failure to observe a temperature dependence for complex formation between RNAP and the Mismatch DNA would reflect the thermodynamics of both a conformational change in the RNAP and the interaction of RNAP with single-stranded DNA.

The interaction of RNAP with single-stranded DNA may promote the transition of the complex to a heparin-resistant form (e.g. the I<sub>1</sub> to I<sub>2</sub> transition) (28, 29). Eco RNAP additionally requires the presence of double-stranded promoter regions; Eco RNAP-SS DNA complexes are not heparin-resistant. However, Taq RNAP can form heparin-resistant complexes with just the SS DNA, likely reflecting a tighter interaction under comparable conditions, as also shown in Fig. 6A. Thus the interaction with single-stranded DNA may be more important to open complex formation with Taq than with Eco RNAP. Given the low affinities of both RNA polymerases for single-stranded DNA at physiological temperatures, prior weakening of base pairing by some combination of base flipping (4, 27, 37–39) and DNA torquing or bending (38, 40) would be a prerequisite for the initiation of DNA melting.

Conclusions—Through the use of wt and mutant sigma factors, as well as a variety of DNA templates, we have been able to identify differences between Taq and Eco RNAP with respect to the temperature dependence of promoter melting and the roles of amino acids in region 2.3 of sigma. Most striking is the finding that for Eco, but not for Taq RNAP, the effects of the mutations are mitigated by temperature increases. For Eco RNAP, the mutations likely affect both the RP<sub>c</sub> to I<sub>1</sub> and I<sub>1</sub> to I<sub>2</sub> isomerizations in Scheme 1, which reflect two consecutive conformational transitions proposed (10) to explain the temperature dependence of open complex formation. As a result, the jaws of the RNAP move to encircle the downstream region of promoter DNA in the open complex. For Taq RNAP, in contrast to Eco RNAP, the mutations and temperature would act at different steps.

For both fork junction and SS DNA, the interaction with either Taq or Eco RNAP is favored at low temperatures, opposite to that observed with open complex formation at promoters (10). This behavior is an indication that any associated conformational changes would be different from those occurring during open complex formation. Indeed, the forked junction templates lack the downstream DNA postulated (10) to elicit such conformational changes. With these templates, low temperatures can overcome the effects of the mutations in region 2.3 of sigma for both Taq and Eco RNAP.

Taq RNAP binds SS DNA more tightly than Eco RNAP at both 9 and 37 °C. In the absence of interactions with upstream double-stranded DNA, the complexes with Eco RNAP are heparin-sensitive, whereas a significant fraction of the complexes with Taq RNAP survive such a challenge. The biological significance of the observed temperature dependence of the affin-
ity of RNAP for single-stranded DNA may be both avoidance at high temperatures of competing interactions with promoters for RNAP binding and stabilization of the open complex at low temperatures, where DNA reannealing would be greatly favored.

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REFERENCES

1. Record, M. T., Jr., Reznikoff, W. S., Craig, M. L., McQuade, K. L., and Schlax, P. J. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., ed) pp. 792–820, ASM Press, Washington, D.C.
2. deHaseth, P. L., Zupancic, M. L., and Record, M. T., Jr. (1998) J. Bacteriol. 180, 3019–3025
3. Jiang, Y.-L., and Helmann, J. D. (1994) J. Mol. Biol. 235, 1470–1488
4. Fenton, M. S., Lee, S. J., and Gralla, J. D. (2000) EMBO J. 19, 1130–1137
5. Panaghie, G., Aiyar, S. E., Bobb, K. L., Hayward, R. S., and deHaseth, P. L. (2000) J. Mol. Biol. 299, 1217–1230
6. Tomsic, M., Tsujikawa, L., Panaghie, G., Wang, Y., Azok, J., and deHaseth, P. L. (2001) J. Biol. Chem. 276, 31891–31896
7. Malhotra, A., Severinova, E., and Darst, S. A. (1996) J. Mol. Biol. 2603–2609
8. Mallinstra, A., Severinova, E., and Darst, S. A. (1996) Cell 87, 127–136
9. Campbell, E. A., Muzzin, O., Chienov, M., Sun, J.-L., Olson, C. A., Weinman, O., Trester-Zedlitz, M. L., and Darst, S. A. (2000) Mol. Cell 9, 527–539
10. Craig, M. L., Tsodikov, O. V., McQuade, K. L., Schlax, P. E., Jr., Capp, M. W., Saecker, R. M., and Record, M. T., Jr. (1998) J. Mol. Biol. 283, 741–756
11. Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K., and Darst, S. A. (1999) Cell 96, 811–824
12. Murakami, K. S., Masuda, S., Campbell, E. A., Muzzin, O., and Darst, S. A. (2002) Science 296, 1285–1290
13. Murakami, K. S., Masuda, S., and Darst, S. A. (2002) Science 296, 1280–1284
14. Vassylyev, D., Békine, S.-I., Laptenko, O., Lee, J., Vassylyeva, M. N., Burkhov, S., and Yokoyama, S. (2002) Nature 417, 712–719
15. Meier, T., Schink, P., Wedel, A., Cellai, L., and Heumann, H. (1995) Nucleic Acids Res. 23, 988–994
16. Minakhin, L., Nechaev, S., Campbell, E. A., and Severinov, K. (2001) J. Bacteriol. 183, 71–76
17. Kuznetsov, K., Minakhin, L., and Severinov, K. (2003) Methods Enzymol. 370, 94–108
18. Xue, Y., Hogan, B. P., and Erie, D. A. (2000) Biochemistry 39, 14356–14362
19. Aiyar, S. E., Jiang, Y.-L., Helmann, J. D., and deHaseth, P. L. (1994) Biochemistry 33, 11501–11506
20. deHaseth, P. L., and Tsujikawa, L. (2003) Methods Enzymol. 370, 553–567
21. Maseda, H., and Hoshino, T. (1995) FEMS Microbiol. Lett. 128, 127–134
22. Sasse-Dwight, S., and Gralla, J. D. (1989) J. Biol. Chem. 264, 8074–8081
23. Lozinski, T., and Wierzchowski, K. L. (2003) Anal. Biochem. 320, 239–251
24. Mangel, W. F., and Chamberlin, M. J. (1974) J. Biol. Chem. 249, 3007–3013
25. Kuilbachinskii, A., Bass, I., Bogdanova, E., Goldfarb, A., and Nikiforov, V. (2004) J. Biol. Chem. 280, 7818–7820
26. Guo, Y., and Gralla, J. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11655–11660
27. Helmann, J. D., and Chamberlin, J. (1988) Annu. Rev. Biochem. 57, 859–872
28. Tsujikawa, L., Tsodikov, O. V., and deHaseth, P. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3493–3498
29. Fenton, M. S., and Gralla, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9020–9025
30. Marr, M. T., and Roberts, J. W. (1997) Science 276, 1258–1260
31. Huang, X., Lopez de Saro, F. J., and Helmann, J. D. (1997) Nucleic Acids Res. 25, 2603–2609
32. Callaci, S., and Heyduk, T. (1996) Biochemistry 35, 3312–3320
33. Fedoriw, A. M., Liu, H., Anderson, V. E., and deHaseth, P. L. (1998) Biochemistry 37, 11971–11979
34. Kumar, S., and Nussinov, R. (2001) Cell. Mol. Life Sci. 58, 1216–1233
35. Fenton, M. S., and Gralla, J. D. (2003) J. Biol. Chem. 278, 39669–39674
36. Sun, L., Dove, S. L., Panaghie, G., deHaseth, P. L., and Hochschild, A. (2004) J. Mol. Biol. 343, 1171–1182
37. Tsujikawa, L., Strainie, M., Winqrob, H., Barkley, M., and deHaseth, P. (2002) Biochemistry 41, 15334–15344
38. Young, B. A., Gruber, T. M., and Gross, C. A. (2004) Science 303, 1382–1384
39. Helmann, J. D., and deHaseth, P. L. (1999) Biochemistry 37, 5959–5967
40. deHaseth, P. L., and Helmann, J. D. (1995) Mol. Microbiol. 16, 817–824