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Distinct Functions of Regions 1.1 and 1.2 of RNA Polymerase σ Subunits from *Escherichia coli* and *Thermus aquaticus* in Transcription Initiation*[S]

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Background: RNA polymerases (RNAPs) from *Thermus aquaticus* and *Escherichia coli* differ in many aspects of transcription initiation.
Results: Regions 1.1 and 1.2 of the σ subunit determine instability and cold sensitivity of promoter complexes of *T. aquaticus* RNAP.
Conclusion: Substitutions in σ regions 1.1 and 1.2 modulate RNAP-promoter interactions.
Significance: Evolutionary changes in the σ subunit determine functional differences between bacterial RNAPs during transcription initiation.

RNA polymerase (RNAP) from thermophilic *Thermus aquaticus* is characterized by higher temperature of promoter opening, lower promoter complex stability, and higher promoter escape efficiency than RNAP from mesophilic *Escherichia coli*. We demonstrate that these differences are in part explained by differences in the structures of the N-terminal regions 1.1 and 1.2 of the *E. coli* σ^{70} and *T. aquaticus* σ^{A} subunits. In particular, region 1.1 and, to a lesser extent, region 1.2 of the *E. coli* σ^{70} subunit determine higher promoter complex stability of *E. coli* RNAP. On the other hand, nonconserved amino acid substitutions in region 1.2, but not region 1.1, contribute to the differences in promoter opening between *E. coli* and *T. aquaticus* RNAPs, likely through affecting the σ subunit contacts with DNA nucleotides downstream of the −10 element. At the same time, substitutions in σ regions 1.1 and 1.2 do not affect promoter escape by *E. coli* and *T. aquaticus* RNAPs. Thus, evolutionary substitutions in various regions of the σ subunit modulate different steps of the open promoter complex formation pathway, with regions 1.1 and 1.2 affecting promoter complex stability and region 1.2 involved in DNA melting during initiation.

The σ subunit of bacterial RNAP[3] has been implicated in various steps of transcription initiation, from promoter recognition and melting to initiation of RNA synthesis and promoter escape (1–6). All primary σ subunits (σ^{70} in *Escherichia coli*; σ^{A} in other bacteria) that are involved in transcription of housekeeping genes contain four conserved regions, further divided into subregions (7, 8). Structural analyses of *E. coli* (*Eco*) σ^{70} (9) and *Thermus aquaticus* (*Taq*) σ^{A} (8) subunits, as well as *Taq* (3) and closely related *Thermus thermophilus* (4) RNAP holoenzymes revealed that σs contain four domains, σ1.1 (comprised of region 1.1), σ2 (regions 1.2 through 2.4), σ3 (regions 3.0–3.2), and σ4 (regions 4.1–4.2). Based on genetic, biochemical, and structural studies, the σ regions were assigned various functions in transcription initiation. In particular, regions 4.2, 2.3–2.4, and 3.0 were implicated in recognition of the −35, −10, and TG (extended −10) promoter elements, respectively (reviewed in Refs. 1 and 2). Region 1.2 in *Eco* σ^{70} and *Taq* σ^{A} was shown to recognize an additional promoter element (GGGA motif in *Taq*) located downstream of the −10 element (10, 11).

Recently, the three-dimensional structure of domain 2 of the *Taq* σ^{A} subunit in complex with a short DNA oligonucleotide containing the −10 element was determined (12). In this complex the nontemplate DNA strand forms tight contacts with σ region 2 (Fig. 1*A*), incompatible with double-stranded DNA conformation, explaining previously established roles of region 2 in promoter recognition, DNA melting, and stabilization of RNAP-DNA interactions (1, 13–17). Although the exact position of the nontemplate DNA strand downstream of the −10 element remains unknown, structural modeling, site-specific cross-linking, and biochemical analyses suggest that this DNA segment may directly contact σ region 1.2, depending on the DNA sequence context (10, 11, 18, 19). Amino acid substitutions at various positions of region 1.2 in *Eco* σ^{70} were shown to decrease RNAP activity, inhibit promoter DNA melting, and destabilize the open promoter complex (Fig. 1*B*) (19, 20). In addition to its direct role in DNA recognition, region 1.2 was shown to allosterically modulate the DNA binding activity of region 2 (21).

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[S] This article contains supplemental references and Fig. S1.
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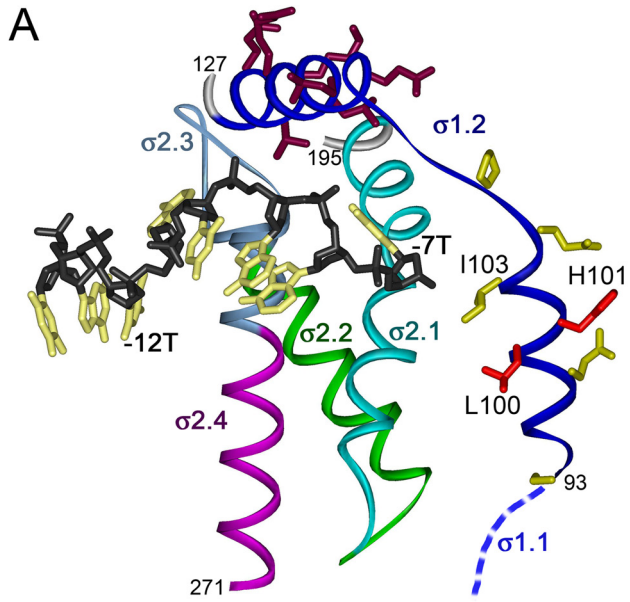
[3] The abbreviations used are: RNAP, RNA polymerase; *Eco*, *E. coli*; *Taq*, *T. aquaticus*.



B

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                  ******
                  S  H  V      MM    K   P PA  P
Eco     92  GRTTDPVRMYMREMGTVELLTREGEIDIAKRIEDGIN  128
Bsu     96  VKIN.....LK.I.R.N..SAKE..AY.QK..E.DE  132
Aae     84  S.DG...KL.L....KIP..K..E..YY.RQ..I.RK  120
Tma    114  SNVR.SIK..LK.I.KIP...PAQ.REL.R.AQM---  147
Rpr     99  .S.D....L.LKD..G.D.....N.VE......E.RK  135
Dra    161  AVSN....Q.LH.I.R.S...L.E..AL.R...E.EE  197
Tth     75  IS.S....Q.LH.I.Q.P...L.E.VEL.RKV.E.ME  111
Taq     90  VS.S....Q.LH.I.Q.P...L.E...L.RKV.E.ME  126
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C

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T7A1
GTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCATCGAGAGGGACACGGCG
T7A1cons
GTATTGACTTAAAGTCTAACCTATAGTATAATTACAGCCATCGAGAGGGACACGGCG
T7A1_TGcons
GTATTGACTTAAAGTCTAACCTATGGTATAATTACAGCCATCGAGAGGGACACGGCG
λ P_R
GTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCATGTAGTAAGGAGGTTGTA
lacUV5
GCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAA
   -35                      -10        +1
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FIGURE 1. **Recognition of the nontemplate promoter strand by the σ subunit and sequence comparisons of σ region 1.2 from various bacteria.** *A*, the structure of *Taq* σ^A domain 2 (amino acids 93–271) in complex with the −10 DNA element (Protein Data Bank code 3UGO) (12). The nonconserved spacer between regions 1.2 and 2.1 is not shown on the structure. The DNA segment containing the −10 element (TGTACAAT) is shown in *black* (backbone) and *light yellow* (bases), and the first (−*12T*) and the last (−*7T*) thymines of the −10 element are indicated. Region 1.2 (*dark blue*) connects to region 1.1 (*blue*), the position of which remains unknown. Amino acids substituted in σ^A region 1.2 in comparison with the *Eco* σ^{70} subunit are shown in *yellow* (first α helix) and *dark violet* (second α helix). Residues Leu-100 and His-101 that probably contact DNA nucleotides downstream of the −10 element are shown in *red*. *B*, sequences of region 1.2 of primary σ subunits from various bacteria: *Bsu*, *Bacillus subtilis*; *Aae*, *Aquifex aeolicus*; *Tma*, *T. maritima*; *Rpr*, *Rickettsia prowazekii*; *Dra*, *Deinococcus radiodurans*; *Tth*, *T. thermophilus*. Region 1.2 is *boxed*; amino acid numbers are indicated at both sides of the alignment. Mutations of conserved amino acids in *Eco* σ^{70} that were shown to strongly affect promoter-dependent activity of RNAP (20) are shown above the *Eco* σ^{70} sequence. Those mutations that were demonstrated to impair promoter opening or promoter escape are *shadowed* and *underlined*, respectively. The positions of alanine substitutions that were proposed to decrease promoter complex stability by affecting σ^{70} contacts with DNA downstream of the −10 element are indicated with *asterisks* (19). Amino acid substitutions in *Taq* σ^A region 1.2 are colored in the same way as in *A*. *C*, sequences of promoters used in this study (only nontemplate strands are shown). The −35, TG, and −10 elements are shown in *blue*, *green*, and *red*, respectively; the +1 nucleotide is shown in *bold italics*.

The DNA binding activity of primary σ subunits is inhibited in free σs, in part because of the presence of a weakly conserved region 1.1 at their N terminus. Removal of this region in *Eco* σ^{70} increases specific interactions of σ^{70} with promoters (22, 23). As revealed by site-specific cross-linking, in *Thermotoga maritima* σ^A region 1.1 is physically close to the promoter recognition regions 2 and 4 (24). Although the structures of regions 1.1 in *Eco* and *Taq* σ subunits remain unknown, the structure of region 1.1 from *T. maritima* was solved by NMR showing that it folds into three α helixes and possesses a negative electrostatic potential on its surface (supplemental Fig. S1) (24). The presence of a high number of negatively charged amino acids is also a characteristic feature of region 1.1 in other primary σ subunits, including *Taq* and *T. thermophilus* σ^A subunits that have highly divergent region 1.1 sequences (supplemental Fig. S1). In addition to the proposed functions in preventing interactions of free σ with DNA, region 1.1 likely plays an important role in holoenzyme assembly and open complex formation (25–28). Several studied amino acid substitutions and deletions in this region in *Eco* σ^{70} (supplemental Fig. S1) decreased RNAP activity, impaired open complex formation, and/or decreased the stability of promoter complexes (25, 27–29).

Although a wealth of biochemical and genetic data on promoter recognition and transcription initiation were accumulated for *Eco* RNAP, most structural information on the mechanisms of bacterial transcription was obtained for RNAPs from thermophilic *Taq* and *T. thermophilus* that are distantly related to *Eco*. Although all multisubunit RNAPs share a highly conserved architecture, *Thermus* RNAPs significantly differ from the *Eco* RNAP in structural details (30–33) and transcription properties (18, 34–39). In particular, *Taq* RNAP has a higher temperature optimum of activity than the *Eco* RNAP and is inactive at low and moderate temperatures. These differences were shown to be associated with the catalytic properties of *Taq* core RNAP and with the properties of the *Taq* σ^A subunit that is unable to induce promoter DNA melting at temperatures below 45 °C (36–38, 40). The cold sensitivity of promoter opening by the *Taq* σ^A subunit in comparison with the *Eco* σ^{70} subunit was explained by substitutions of nonconserved amino acids in region 2 and by differences in the structures of the N-terminal parts of these σ subunits (36–38). However, individual roles of σ^A regions 1.1 and 1.2 in transcription initiation by *Taq* RNAP were not investigated.

A characteristic feature of most studied promoter complexes of *Eco* RNAP is their very high stability *in vitro*, with half-lives ranging from tens of minutes to hours. In contrast, promoter complexes of *Taq* RNAP were shown to be intrinsically unstable and to dissociate within seconds (10, 36). The structural features of *Taq* RNAP that can explain the low stability of promoter complexes formed by this RNAP remain unidentified.

In this work we extended analysis of functional differences between *Eco* and *Taq* RNAPs in transcription initiation, with particular emphasis on the roles of regions 1.1 and 1.2 of the σ^{70} and σ^A subunits in promoter complex formation. We demonstrated that both regions 1.1 and 1.2 of the σ^A subunit determine the low stability of promoter complexes of *Taq* RNAP, whereas substitutions in region 1.2 of σ^A contribute to the cold sensitivity of promoter opening by *Taq* RNAP, suggesting pos-

sible functions of these regions at different steps of the open complex formation pathway.

EXPERIMENTAL PROCEDURES

RNAPs and Promoters—Wild-type *Eco* and *Taq* core RNAPs were purified from *E. coli* BL21(DE3) cells overproducing all four core RNAP subunits from plasmids pVS10 and pET28ABCZ, respectively, as described previously (35, 36, 41). Genes coding for mosaic *Eco* and *Taq* σ subunits were generated by PCR mutagenesis of wild-type *rpoD* genes and cloned between NdeI and EcoRI sites into the pET28 plasmid. Wild-type *Eco* σ^{70} and *Taq* σ^{A} subunits and mosaic σ subunits, all containing His_6 tags at their N termini, were overexpressed in *E. coli* BL21(DE3) and purified as described in Refs. 34 and 36.

Promoter DNA fragments for *in vitro* transcription were obtained as follows. The T7A1, T7A1cons, and T7A1_TGcons promoters (positions from −85 to +53 nucleotides relative to the starting point of transcription) were obtained by PCR from synthetic oligonucleotide templates. The *lac*UV5 promoter (positions −59 to +58) was obtained as described in Ref. 21. The λP_R promoter fragment (positions −81 to +54) was obtained by PCR from plasmid pIA226. The sequences of all promoters are shown on Fig. 1*C*.

In Vitro Transcription—Transcription assays were performed in transcription buffer containing 40 mM Tris-HCl, pH 7.9, 40 mM KCl, and 10 mM $MgCl_2$. Holoenzyme RNAPs were prepared by incubating core RNAP (final concentration, 100 nM) and either the wild type or mosaic σ subunits (500 nM) in the transcription buffer for 5 min at 25 °C. DNA template was added (10–30 nM), and the samples were incubated for 3–5 min at desired temperatures (45 °C for holoenzymes containing *Eco* core RNAP and 55 °C for holoenzymes containing *Taq* core RNAP in most experiments). For analysis of promoter complex stabilities on T7A1 and λP_R promoters, heparin was added to 10 μg/ml. Following incubation of the samples for different time intervals at the same temperatures, transcription reactions were initiated by the addition of dinucleotide primer CpA (25 μM) and UTP (10 μM, with the addition of [α-^{32}P]UTP). The reactions were stopped after 1 min by the addition of an equal volume of buffer containing 8 M urea and 20 mM EDTA; the 3-nucleotide RNA products were analyzed by 23% denaturing PAGE followed by phosphorimaging. For analysis of temperature dependence of transcription on the *lac*UV5 promoter, reactions were performed in the presence of trinucleotide primer ApApU and UTP (with the addition of [α-^{32}P]UTP); the samples were transferred to desired temperatures 5 min prior to addition of nucleotide substrates. For analysis of promoter escape, the reactions were performed in transcription buffer containing 100 mM KCl; all four nucleotide substrates were added (100 μM of ATP, CTP, and GTP and 10 μM of UTP with the addition of [α-^{32}P]UTP), either in the absence or in the presence of the CpA primer (25 μM). The transcription reactions were stopped after 5 min, and RNA products were separated by 20% denaturing PAGE.

$KMnO_4$ Footprinting—For the $KMnO_4$ footprinting experiments, the *lac*UV5 promoter was labeled at the 3′-end of the template DNA strand with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP as described in Refs. 36 and 42. Holoenzyme RNAPs (100 nM core and 500 nM σ) were incubated with the labeled DNA fragment (10 nM) in the transcription buffer for 10 min at either 25 or 45 °C, followed by the addition of $KMnO_4$ to 2 mM. The reaction was stopped after 20 s by the addition of equal volume of solution containing 1 M β-mercaptoethanol and 1 M sodium acetate. DNA was ethanol-precipitated, treated with piperidine as described (6), and analyzed on 10% denaturing polyacrylamide gel.

Nontemplate Oligonucleotide Binding and Cross-linking—Apparent dissociation constants (K_d) for the oligonucleotide binding to RNAP holoenzymes were determined by nitrocellulose filtration method (43). 5′-End-labeled nontemplate promoter oligonucleotide containing the −10 element (see Fig. 5*C*), taken at fixed 0.03 nM concentration, was mixed with RNAP holoenzyme, taken at varying concentrations (250 nM σ plus 0.1–100 nM core RNAP), in binding buffer containing 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 100 mM NaCl; incubated for 10 min at 25 °C; and filtered through 0.45-μm nitrocellulose filters (HAWP, Millipore), followed by phosphorimaging. The binding curves were fit to hyperbolic equation $B = B_{max}*[RNAP]/([RNAP] + K_d)$, where B is a fraction of bound DNA, B_{max} is the maximum binding, and K_d is the apparent dissociation constant, using GraFit software (Erithacus Software). RNAP-DNA cross-linking experiments were performed in buffer containing 40 mM Hepes, pH 8.0, 5 mM $MgCl_2$, and 100 mM NaCl as described (44). Core RNAP, σ subunits, and nontemplate oligonucleotide were taken at 50, 300, and 10 nM, respectively. After incubation for 10 min at 25 °C, the samples were irradiated for 10 min with a 254-nm UV lamp (4 watts; Spectroline). The DNA-protein complexes were separated by 5% SDS-PAGE.

RESULTS

The Instability of Taq RNAP Promoter Complexes Is Determined by the N Terminus of the σ^A Subunit—To determine which component of the *Taq* RNAP holoenzyme determines the instability of promoter complexes formed by this RNAP in comparison with the *Eco* RNAP holoenzyme, we compared stabilities of complexes formed on the T7A1 promoter by wild-type *Eco* and *Taq* RNAPs and by a hybrid RNAP containing *Eco* core and *Taq* σ^A (*Eco*/*Taq*). It should be noted that the properties of promoter complexes formed by a reciprocal hybrid holoenzyme containing *Taq* core and *Eco* σ^{70} could not be tested because such holoenzyme is inactive (35, 36). For each of the three RNAPs, we measured the kinetics of promoter complex dissociation in the presence of heparin (Fig. 2*A*). The measurements were performed at 45 °C in the case of *Eco* and hybrid *Eco*/*Taq RNAPs* and at 55 °C in the case of *Taq* RNAP. In agreement with published data, we found that promoter complexes of *Eco* RNAP were stable with half-life exceeding 10 min. In contrast, promoter complexes of *Taq* RNAP were unstable and almost completely dissociated within 20 s. The hybrid *Eco*/*Taq* holoenzyme also displayed much lower stability of promoter complexes, with a half-life of about 40 s (Fig. 2*A*). Importantly, the hybrid holoenzyme was highly active and fully melted promoter DNA around the starting point of transcription under the same conditions (at 45 °C), demonstrating that the low promoter complex stability does not result from its

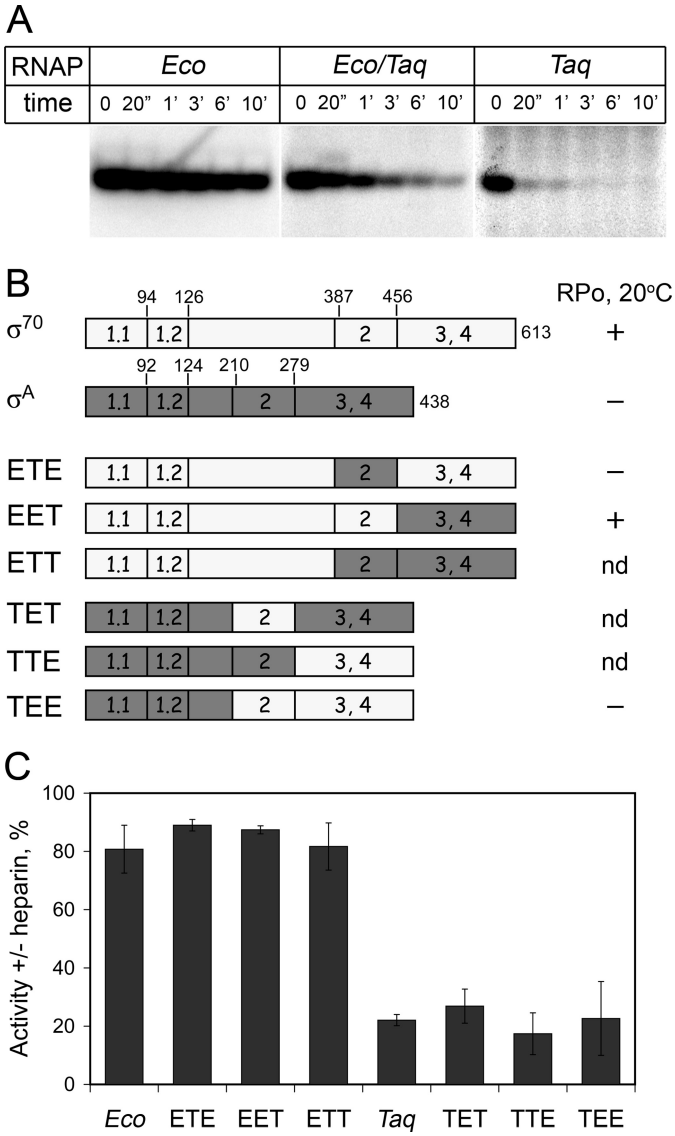

FIGURE 2. **Stabilities of promoter complexes formed by RNAPs containing *Eco*, *Taq* σ subunits, and their mosaic variants.** *A*, stabilities of promoter complexes formed by *Eco*, *Taq*, and hybrid *Eco*/*Taq* RNAPs on the T7A1 promoter measured in the reaction of abortive synthesis in the presence of heparin (10 μg/ml). *B*, schematics of mosaic σ subunits with substitutions of regions 1, 2, and 3/4. The numbers above the schemes correspond to amino acid positions at the borders of conserved regions that were used for exchanging σ segments. The ability of various σ subunits to induce formation of the open promoter complex by *Eco* RNAP at 20 °C is indicated at *right* (the data from Ref. 38). *nd*, no data. *C*, relative stabilities of promoter complexes formed by holoenzymes containing *Eco* core and mosaic σ subunits. The plot shows RNAP activities measured after 3′ incubation of preformed promoter complexes with heparin relative to activities measured in the absence of heparin (averages and standard deviations from three independent experiments).

inability to form the open promoter complex (36, 38) (see also Fig. 5). In addition to the heparin challenge experiments, we analyzed activity of the T7A1 promoter complexes formed by the three RNAPs under various ionic strength conditions. Promoter complexes of *Taq* RNAP were much more salt-sensitive than promoter complexes of *Eco* RNAP, and promoter complexes of the hybrid RNAP displayed intermediate salt sensitivity.[4] Thus, we conclude that the low stability of promoter complexes of *Taq* RNAP holoenzyme is in a large part determined by the properties of the *Taq* σ^A subunit (but also by the properties of the *Taq* core enzyme, because the *Taq* holoenzyme has lower promoter complex stability than the hybrid *Eco*/*Taq* holoenzyme).

To determine which region of the σ^A subunit is responsible for instability of promoter complexes formed by the hybrid RNAP, we tested several mosaic σ subunits consisting of various parts of the σ^{70} and σ^A subunits. The exchanged segments of the σ subunits included the N-terminal part, including conserved region 1 and a nonconserved spacer between regions 1 and 2 (amino acids 1–386 and 1–209 in σ^{70} and σ^A, respectively); conserved region 2 (amino acids 387–455 and 210–278 in σ^{70} and σ^A, respectively); and the C-terminal part, including conserved regions 3 and 4 (amino acids 456–613 and 279–438 in σ^{70} and σ^A, respectively). In total, six mosaic σ subunits with all possible combinations of these segments (ETE, EET, ETT, TET, TTE, and TEE) were studied (Fig. 2*B*). All of the mosaic subunits were shown to form active holoenzymes with *Eco* core RNAP (38).[5] It was found that RNAP holoenzymes that contained the σ subunits with the N-terminal part from σ^{70} (ETE, EET, and ETT) formed stable promoter complexes, whereas holoenzymes that contained the σ subunits with the N-terminal part from σ^A formed unstable complexes when challenged with heparin (Fig. 2*C*). Importantly, the promoter complex stabilities of RNAPs containing mosaic σs did not correlate with their abilities to form the open promoter complex at low temperatures (20 °C) (Fig. 2*B*). In particular, the holoenzyme containing the ETE σ subunit displayed cold sensitivity of promoter opening (38) but formed stable promoter complexes.

σ Regions 1.1 and 1.2 Together determine the Differences in Promoter Complex Stabilities between Eco and Taq RNAPs—To more precisely locate the region(s) in the N-terminal part of the σ subunit that can affect the promoter complex stability in *Eco* and *Taq* RNAPs, we designed a second set of mosaic σ subunits with substitutions of regions 1.1 and 1.2. The mosaic σ subunits were based either on the σ^{70} or the σ^A sequence and contained replacements of region 1.1 (σ teE, amino acid residues 1–93 in σ^{70} replaced with residues 1–91 from σ^A; σ etT, residues 1–91 in σ^A replaced with residues 1–93 from σ^{70}), region 1.2 (σ etE, residues 94–125 in σ^{70} replaced with residues 92–123 from σ^A; σ teT, residues 92–123 in σ^A replaced with residues 94–125 from σ^{70}), or both (σ ttE that contained regions 1.1 and 1.2 from σ^A) (Fig. 3*A*). Whereas regions 1.1 in σ^{70} and σ^A significantly differ in their sequences, region 1.2 is highly conserved and contains only 14 substitutions in σ^A in comparison with σ^{70} (Fig. 1*B*). Thus, to reveal possible effects of individual substitutions in region 1.2, we obtained a σ^{70} mutant that contained two σ^A-specific substitutions, M102L and R103H (σ MR-LH), that changed amino acids likely involved in contacts with the nontemplate DNA strand downstream of the −10 element (see Introduction and Fig. 1).

Analysis of the T7A1 promoter complex stabilities of RNAPs containing *Eco* core RNAP and σ^{70}-based mosaic σ subunits

[4] N. Miropolskaya and A. Kulbachinskiy, unpublished data.

[5] A. Kulbachinskiy, unpublished data.

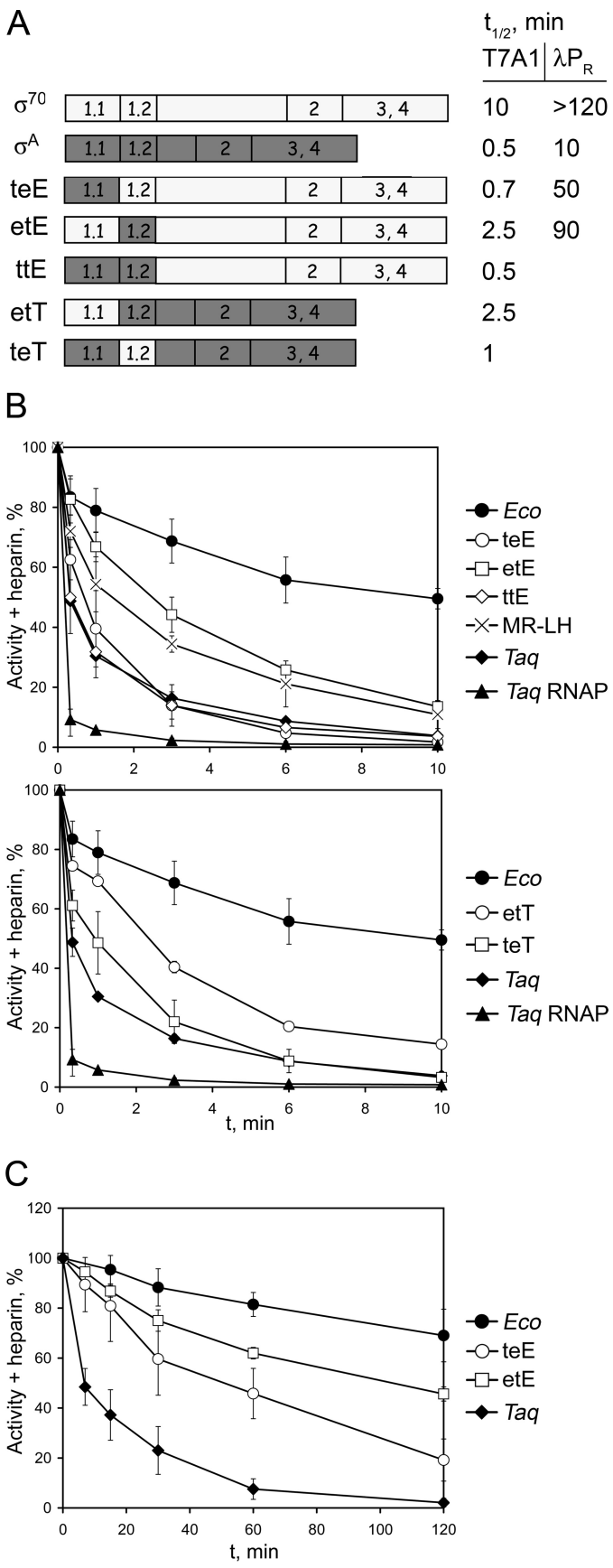

FIGURE 3. **Effects of substitutions of regions 1.1 and 1.2 in the *Eco* and *Taq* σ subunits on promoter complex stability.** *A*, schematics of *Eco* σ^{70}, *Taq* σ^{A}, and mosaic σ subunits with substitutions of regions 1.1 and 1.2. The half-life times of complexes formed by holoenzyme RNAPs containing *Eco* core RNAP and corresponding σ subunits on the T7A1 and λP_R promoters, measured in the presence of heparin (10 μg/ml), are shown on the *right*. *B*, kinetics of dissociation of the T7A1 promoter complexes formed by *Eco*, *Taq* RNAPs, and RNAP holoenzymes containing *Eco* core RNAP and various σ subunits. The data for σ^{70}- and σ^{A}-based subunits are shown on the *upper* and *lower plots*, respectively. RNAP activities were measured after incubation of promoter complexes in the presence of heparin for various time intervals. For each time point, the level of heparin-resistant activity relative to the activity measured in the absence of heparin is shown. *C*, kinetics of dissociation of the λP_R promoter complexes formed by RNAP holoenzymes containing *Eco* core RNAP and various σ subunits.

revealed that the substitution of region 1.1 in the teE σ subunit had the most significant effect on promoter complex stability, measured in the presence of heparin, and decreased it almost to the level of the wild-type σ^{A} subunit (Fig. 3*B*, *upper panel*). Substitution of region 1.2 in σ etE also decreased promoter complex stability but to a lesser extent. Importantly, the effect of the MR → LH substitution in region 1.2 was comparable with the effect of the substitution of the whole region 1.2. Finally, RNAP containing σ ttE with substitutions of both regions 1.1 and 1.2 displayed the same promoter complex stability as RNAP containing wild-type σ^{A} (Fig. 3*B*). Thus, substitutions in these two regions can fully explain the lower stability of promoter complexes formed by the σ^{A}-containing RNAP on the T7A1 promoter.

We then tested the effects of substitutions in the σ^{A}-based σ etT and σ teT subunits on the promoter complex stability. Substitution of region 1.2 in σ teT slightly increased the stability of promoter complexes in comparison with σ^{A} *Taq*. At the same time, substitution of region 1.1 in σ etT had a stronger stabilizing effect on promoter complexes (Fig. 3*B*, *lower panel*).

To test whether the observed effects are general for various promoters, we repeated the experiment with holoenzymes containing *Eco* core RNAP and various σ subunits on the λ P_R promoter that forms more stable complexes with *Eco* RNAP (Fig. 3*C*). Promoter complexes formed by RNAP containing wild-type σ^{70} were highly resistant to heparin challenge and did not dissociate within 120 min. In contrast, the dissociation kinetics was much faster in the case of σ^{A}-containing RNAP ($t_{½}$ = ~10 min). The half-life times of promoter complexes formed by holoenzymes containing mosaic subunits were also decreased in comparison with wild-type *Eco* RNAP, although the effects were less dramatic than in the case of the T7A1 promoter. In particular, the promoter complex half-lives for RNAPs containing σ teE and σ etE were ~50 and ~90 min, respectively (Fig. 3*C*). Thus, it can be concluded that substitutions of regions 1.1 and 1.2 from the σ^{A} subunit destabilize complexes formed by *Eco* RNAP on various promoters.

Region 1.1 from σ^{70} Can Stabilize Promoter Complexes Formed by Taq RNAP—To determine whether substitutions of regions 1.1 and 1.2 could also affect the stability of promoter complexes formed by *Taq* RNAP, we analyzed the dissociation kinetics of promoter complexes formed by RNAPs containing *Taq* core and σ subunits etT and teT. In contrast to σ^{70}, these two mosaic σ subunits were shown to form fully active holoenzymes with *Taq* core RNAP. In the case of the T7A1 promoter, promoter complexes formed by holoenzymes containing both mosaic σ subunits were unstable and rapidly dissociated after

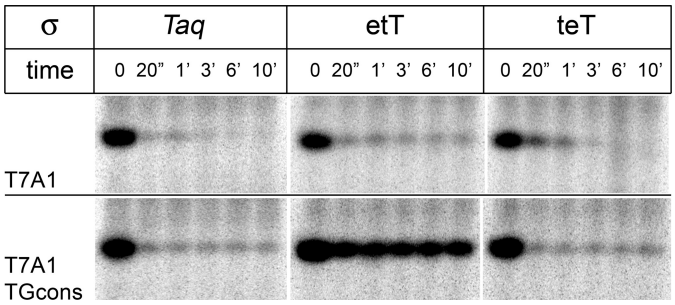

FIGURE 4. **Heparin challenge of promoter complexes formed by *Taq* RNAP holoenzymes containing either wild-type σ^A or mosaic etT and teT σ subunits.** RNAP activities were measured at different time intervals after the addition of heparin to preformed T7A1 (*top panel*) or T7A1TGcons (*bottom panel*) promoter complexes at 55 °C.

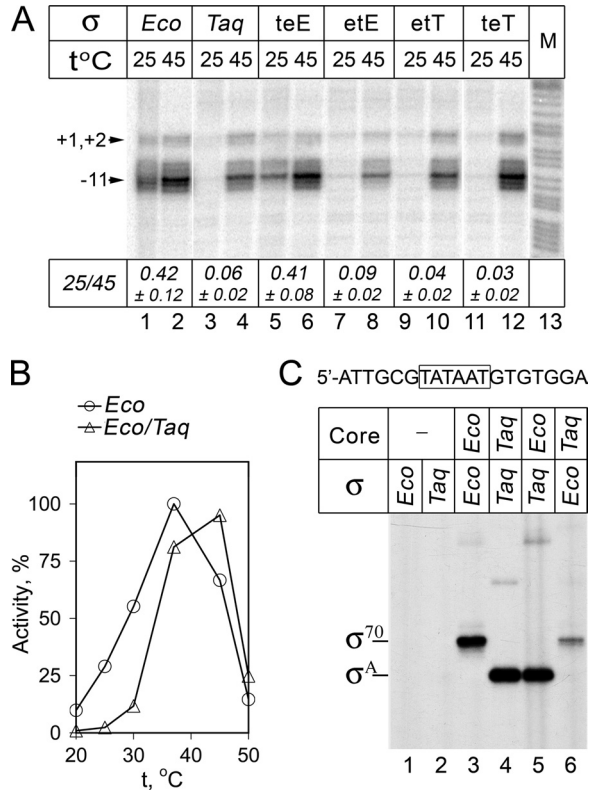

FIGURE 5. **Promoter recognition and opening by RNAP holoenzymes containing *Eco* core and various σ subunits.** *A*, $KMnO_4$ probing of the *lac*UV5 promoter complexes. Footprinting was performed at either 25 or 45 °C on a linear DNA fragment containing the *lac*UV5 promoter; positions of modified thymines in the template promoter strand relative to the starting point of transcription are shown. *Lane 13* contains an A+G cleavage marker. The ratio of modification efficiencies at 25 and 45 °C (measured for position −11) for each RNAP is indicated below the figure (averages and standard deviations from two-three independent experiments). *B*, activities of wild-type *Eco* and hybrid *Eco/Taq* RNAPs measured in the reaction of abortive synthesis on the *lac*UV5 promoter DNA fragment at different temperatures (in percent of the maximal *Eco* RNAP activity). *C*, cross-linking of the nontemplate promoter oligonucleotide (shown on the *top*, the −10 element is *boxed*) with *Eco*, *Taq*, and hybrid *Eco/Taq* RNAP holoenzymes. The experiment was performed at 25 °C. The positions of cross-linked complexes containing the σ^{70} and σ^A subunits are indicated on the *left*.

the addition of heparin (Fig. 4). We then repeated the experiment on promoter T7A1_TGcons, a variant of the T7A1 promoter containing three consensus promoter elements (−10, TG, and −35 elements; Fig. 1*C*), to increase the strength of RNAP-promoter interactions. On this promoter, holoenzymes containing σ^A and σ teT also formed unstable complexes. However, holoenzyme containing σ etT displayed increased promoter complex stability, and a significant fraction of complexes remained active even after 10 min of incubation with heparin (Fig. 4). Thus, substitution of region 1.1 in σ^A with region 1.1 from σ^{70} can stabilize promoter complexes formed by *Taq* RNAP.

Cold Sensitivity of Transcription by Hybrid Eco/Taq RNAP—Previously, hybrid RNAP containing *Eco* core and *Taq* σ^A was shown to be unable to open promoters at moderate temperatures (20–25 °C), suggesting that the σ^A subunit is responsible for the cold sensitivity of promoter opening by *Taq* RNAP (see Introduction and Refs. 36 and 38). We compared DNA melting by the wild-type *Eco* and hybrid *Eco/Taq* RNAP holoenzymes on the model *lac*UV5 promoter by the $KMnO_4$ footprinting and confirmed that, in contrast to the *Eco* holoenzyme, the hybrid holoenzyme was unable to open the promoter at 25 °C (Fig. 5*A*, *lanes 1* and *3*). At the same time, both holoenzymes fully opened the transcription bubble at 45 °C (Fig. 5*A*, *lanes 2* and *4*). Similarly, the hybrid holoenzyme was inactive in *lac*UV5-promoter-dependent transcription at temperatures below 30 °C but synthesized RNA as efficiently as wild-type *Eco* RNAP at higher temperatures (37–45 °C) (Fig. 5*B*).

To confirm that *Taq* σ^A is able to bind *Eco* core RNAP and recognize promoter DNA at low temperatures, we analyzed RNAP interactions with short oligonucleotides corresponding to the nontemplate promoter strand and containing the −10 element (Fig. 5*C*). Previously, recognition of such oligonucleotides by holoenzyme RNAP was shown to mimic the recognition of the −10 element in the open promoter complex (44–46). We found that both wild-type *Eco* and hybrid *Eco*/*Taq* holoenzymes bound the nontemplate oligonucleotide with high affinity, with apparent K_d values of 3.1 ± 1.6 and 3.2 ± 2.6 nM at 25 °C (see "Experimental Procedures" for details on K_d measurements). Furthermore, both RNAPs formed highly efficient cross-links between the corresponding σ subunit and the nontemplate oligonucleotide upon UV irradiation (Fig. 5*C*, *lanes 3* and *5*). Notably, the efficiencies of *Taq* σ^A-oligonucleotide cross-linking were similar in the case of the hybrid *Eco/Taq* RNAP and the *Taq* RNAP holoenzyme (compare *lanes 4* and *5*), suggesting that the *Taq* σ^A subunit similarly interacts with the *Eco* and *Taq* core RNAPs under our experimental conditions. In contrast, no efficient cross-linking was observed in the case of free σ^{70} and σ^A subunits (*lanes 1* and *2*) and in the complex of *Taq* core RNAP and the *Eco* σ^{70} subunit, which do not form an active holoenzyme (*lane 6*). These results suggest that the observed cold sensitivity of promoter opening by the hybrid *Eco/Taq* RNAP is not due to defects in the binding of the heterologous σ^A subunit to the *Eco* core RNAP and/or promoter DNA recognition but likely results from hampered promoter DNA melting at low temperatures.

σ^A-specific Substitutions in Region 1.2 Increase the Temperature of Promoter Opening by Eco RNAP—Previously, it was shown that the cold sensitivity of DNA melting by the *Taq* σ^A subunit is explained by structural features of region 2 and of the N-terminal part of σ^A, including regions 1.1 and 1.2 (38). In particular, substitutions of either region 2 or the N-terminal part in *Eco* σ^{70} with corresponding regions from σ^A (in the

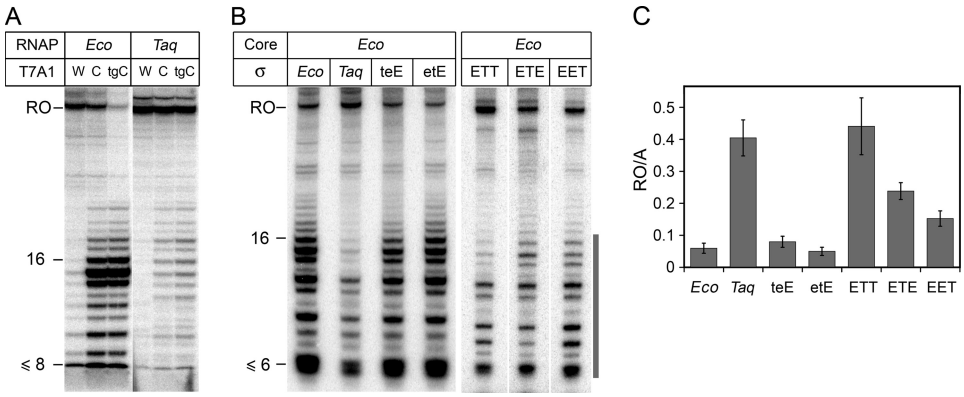

FIGURE 6. **Abortive synthesis and promoter escape by various RNAP holoenzymes.** *A*, transcription by *Eco* and *Taq* RNAPs on wild-type T7A1 (*W*), T7A1cons (*C*), and T7A1_TGcons (*tgC*) promoters. Transcription was performed at either 37 °C (*Eco* RNAP) or 55 °C (*Taq* RNAP) in the presence of all four NTPs. *B*, transcription by *Eco* holoenzymes containing various σ subunits. Transcription was performed at 45 °C on the T7A1_TGcons promoter in the presence of NTPs and dinucleotide primer CpA. *C*, promoter escape efficiencies by RNAP holoenzymes containing *Eco* core RNAP and various σ subunits. For each RNAP, the promoter escape efficiency was determined as the ratio of the full-length (*RO*) to abortive (*A*) RNAs; positions of abortive transcripts used for calculation are indicated by a *vertical gray line* on Fig. 6*B* (the data from two-three independent experiments).

mosaic ETE and TEE σ subunits; Fig. 2*B*) resulted in cold sensitivity of promoter opening by *Eco* RNAP (38). We therefore tested whether substitutions of regions 1.1 and 1.2 in the mosaic σ^{70} and σ^{A} subunits obtained in this work can affect promoter opening by *Eco* RNAP. We found that holoenzymes containing the σ etT and σ teT subunits did not open the *lac*UV5 promoter at 25 °C (*lanes 9–12*), an expected result because these σs contained region 2 from σ^{A} *Taq*, which by itself imposes the cold sensitivity of promoter opening (38). In contrast, the mosaic σ teE subunit with substitution of region 1.1 was able to induce DNA melting at both 25 and 45 °C (*lanes 5* and 6). However, the σ etE subunit with substitution of region 1.2 did not support DNA melting at 25 °C, although it opened the promoter at 45 °C (*lanes 7* and *8*). Thus, σ^{A}-specific substitutions in region 1.2, but not in region 1.1, increase the temperature of promoter opening by *Eco* RNAP. Importantly, RNAP containing the σ etE subunit formed more stable promoter complexes than the σ teE-containing holoenzyme, suggesting that substitutions of regions 1.1 and 1.2 independently affect different promoter complex properties.

Differences between Eco and Taq RNAPs in Abortive Synthesis and Promoter Escape Are Partially Determined by σ Regions 2–4—Changes in stabilities of promoter complexes of *Eco* RNAP were shown to significantly affect the efficiencies of abortive synthesis and promoter escape (47). Because *Taq* RNAP forms much less stable promoter complexes than the *Eco* RNAP, one could expect that it should also differ from *Eco* RNAP in the promoter escape efficiency. We found that this indeed was the case. To analyze promoter escape, we performed transcription on the T7A1 promoter and its two consensus variants, T7A1cons (containing −10 and −35 consensus elements) and T7A1_TGcons (containing −10, TG and −35 elements) (Fig. 1*C*) that are expected to form strong σ-mediated contacts with RNAP. In the case of *Eco* RNAP, large amounts of abortive RNAs of various lengths (up to 16 nucleotides) were synthesized during transcription initiation on the consensus promoters (Fig. 6*A*) (6, 41). Furthermore, the efficiency of the full-length RNA synthesis was significantly decreased in the case of the T7A1_TGcons promoter. In contrast, *Taq* RNAP synthesized much lower amounts of abortive RNAs, although the level of abortive synthesis was increased on the consensus promoters (Fig. 6*A*). *Taq* RNAP was also able to efficiently synthesize the full-length RNA on all three promoter variants.

To determine whether the increased efficiency of promoter escape by *Taq* RNAP can be explained by the properties of the σ^{A} subunit, we analyzed transcription by the hybrid *Eco/Taq* RNAP. In the case of this RNAP, the amounts of abortive RNAs synthesized during initiation were significantly decreased, and the efficiency of promoter escape was increased in comparison with the σ^{70} RNAP holoenzyme (Fig. 6, *B* and *C*). Thus, the σ^{A} subunit promotes more efficient escape to elongation. At the same time, the hybrid RNAP was still less efficient in promoter escape than the *Taq* RNAP holoenzyme, demonstrating that the core enzyme also contributes to the higher promoter escape efficiency displayed by *Taq* RNAP (Fig. 6, compare *A* and *B*).

We then tested whether regions 1.1 and 1.2 of the σ^{70} and σ^{A} subunits contribute to the differences in promoter escape between *Eco* and *Taq* RNAPs. It was found that RNAP holoenzymes containing *Eco* core RNAP and σ teE or σ etE, bearing regions 1.1 or 1.2 from *Taq*, were characterized by the same promoter escape efficiencies as RNAP containing wild-type σ^{70} (Fig. 6, *B* and *C*). In contrast, RNAP containing the σ ETT subunit with regions 2–4 taken from the *Taq* σ^{A} subunit behaved similarly to the σ^{A}-containing RNAP holoenzyme. RNAPs containing σ subunits with individual substitutions of region 2 (σ ETE) or regions 3 and 4 (σ EET) displayed intermediate promoter escape efficiencies (Fig. 6*C*). Thus, substitutions of these regions in *Eco* σ^{70} with the corresponding regions from σ^{A} facilitate promoter escape by RNAP.

DISCUSSION

While sharing a conserved transcription mechanism, RNAPs from different bacteria may significantly differ in transcription properties, as a result of phylogenetic divergence or adaptation to various life conditions, as in the case of mesophilic and thermophilic bacteria. The differences between mesophilic *Eco* and thermophilic *Taq* RNAPs affect various steps of transcription, from promoter recognition and open complex formation to RNA elongation and termination (34, 36–40). In particular, at

the transcription initiation step *Taq* RNAP displays cold sensitivity of promoter opening, lower promoter complex stability and higher promoter escape efficiency in comparison with *Eco* RNAP. In this work, we focused on analysis of the roles of the *Eco* σ^{70} and *Taq* σ^{A} subunits in defining specific differences between the *Eco* and *Taq* RNAPs during initiation. Below, we discuss possible impact of evolutionary variations in the structure of various regions of the σ subunit on different steps of the open complex formation by *Eco* and *Taq* RNAPs.

Formation of the open promoter complex by *Eco* RNAP was previously shown to proceed via at least three intermediates: RP_c, the closed promoter complex with fully double-stranded DNA; intermediate complex I_1, in which the downstream DNA duplex is partially bent and placed inside the DNA binding cleft of RNAP; and complex I_2, containing the open transcription bubble at the starting point of transcription (reviewed in Ref 48). Further isomerization of the unstable I_2 complex into the stable open promoter complex is accompanied by formation of tight contacts of RNAP with the downstream DNA duplex (49–51). Although forming highly stable complexes on most promoters, *Eco* RNAP was found to form unstable complexes on stringent-response promoters, such as the ribosomal *rrnB* P1 promoter (see Ref. 11 and references therein). The *rrnB* P1 promoter complexes were shown to have a shortened downstream DNA footprint (52) and to display high efficiency of promoter escape (53). It was therefore proposed that these complexes may be trapped at the I_2 step of the open complex formation (49). The properties of complexes formed by *Taq* RNAP on most promoters are remarkably similar to the properties of the *rrnB* P1 promoter complexes. Furthermore, *Taq* RNAP also has a shortened downstream footprint on promoter DNA (54). We therefore speculate that the *Taq* RNAP promoter complexes may also correspond to the unstable I_2 intermediate previously described for *Eco* RNAP. However, a detailed kinetic analysis is needed to establish the exact open complex formation pathway by *Taq* RNAP.

We demonstrated that the instability of promoter complexes of *Taq* RNAP in comparison with *Eco* RNAP is determined by both core RNAP and the σ^{A} subunit. The core-dependent variations in promoter complex stability may be probably explained by the differences in RNAP contacts with downstream DNA. In particular, *Taq* core RNAP lacks the SI3 domain that was previously hypothesized to form stabilizing contacts with the downstream DNA duplex in promoter complexes of *Eco* RNAP (49–51, 55). The σ-dependent variations in promoter-complex stability were shown to be mainly determined by σ region 1.1 and, to a lesser extent, region 1.2. Importantly, substitutions of regions 1.1 and 1.2 in the *Eco* σ^{70} and *Taq* σ^{A} subunits affected promoter complex stabilities on both the T7A1 and λP_R promoters, which are characterized by different heparin sensitivities and may differ in the structures of intermediates formed by RNAP holoenzyme during promoter opening (49, 56).

Previously, region 1.1 was proposed to play an important role in the open promoter complex formation, illuminated by deleterious effects of several studied mutations in region 1.1 in the σ^{70} subunit on transcription by *Eco* RNAP (25, 27–29). Based on kinetic analysis and FRET measurements, it was hypothesized that region 1.1 binds within the downstream DNA binding cleft in the free *Eco* RNAP holoenzyme, likely remains bound inside the cleft in the I_1 and I_2 complexes, and is ejected from the cleft upon formation of the tight downstream RNAP-DNA contacts in the open promoter complex (49, 50, 57, 58). Thus, species-specific differences in the structures of σ region 1.1 in *Eco* and *Taq* RNAPs may affect its interactions with core RNAP and downstream DNA in intermediate and open promoter complexes, resulting in changes of their relative stabilities and shifting the equilibrium between them.

Nonconserved amino acids in region 1.2 also contribute to the lower promoter complex stability of *Taq* RNAP. Remarkably, substitution of just two amino acids in *Eco* σ^{70}, M102L and R103H, located in the central part of the first of the two α helixes comprising region 1.2 had the same effect on promoter complex stability as substitution of the whole region 1.2. Thus, these two amino acids likely make the main contribution to the observed differences in promoter complex stabilities determined by σ region 1.2. Previously, alanine substitutions of residues Met-100 through Met-105 in *Eco* σ^{70} (corresponding to residues Gln-98–Ile-103 in σ^{A}; Fig. 1) were shown to destabilize promoter complexes of *Eco* RNAP, likely by disrupting RNAP contacts with the discriminator region located downstream of the −10 element (Fig. 1*B* and Ref. 19). In particular, residue Met-102 of *Eco* σ^{70} (corresponding to Leu-100 in *Taq* σ^{A}; Fig. 1*A*) was proposed to directly interact with DNA two nucleotides downstream of the −10 element and its alanine substitution decreased site-specific cross-linking of region 1.2 with DNA (19). Similarly, the instability of the *rrnB* P1 promoter complexes was proposed to be in part explained by the absence of favorable interactions of σ region 1.2 with the discriminator region at this promoter (11). Thus, nonconserved substitutions in region 1.2 in *Taq* σ^{A} may affect the open complex stability through weakening the σ-DNA contacts. Importantly, however, in contrast to the previously studied alanine substitutions in σ^{70}, σ^{A}-specific substitutions do not disrupt interactions of region 1.2 with DNA, because σ^{A} region 1.2 was shown to specifically recognize the GGGA element downstream of the −10 element in promoter complexes of *Taq* RNAP (10, 18).

In addition to their effects on promoter complex stability, substitutions of nonconserved amino acids in region 1.2 of the σ^{70} subunit with corresponding residues from the σ^{A} subunit resulted in cold sensitivity of promoter opening by *Eco* RNAP, suggesting that region 1.2 is directly involved in DNA melting during initiation. In support of this, mutations in σ^{70} region 1.2 were previously shown to impair promoter opening by *Eco* RNAP holoenzyme (20). Substitutions of nonconserved amino acids located in the first α-helix of region 1.2, including the M102L and R103H (Fig. 1*A*), can likely directly affect interactions of the σ subunit with the nontemplate promoter strand and hinder DNA melting at low temperatures. Thus, both the cold sensitivity and the instability of promoter complexes may result from the loss of favorable contacts of region 1.2 with DNA downstream of the −10 element. In addition, substitutions of other nonconserved residues of region 1.2, not involved in direct contacts with DNA (in particular, in the second α helix of region 1.2), may indirectly affect DNA melting, either by changing σ-core interactions or by affecting the conformation

of domain σ2. In support of this, substitutions of conserved residues in this part of region 1.2 in σ^{70} (shown in Fig. 1*B* above the σ^{70} sequence) also decreased RNAP activity and inhibited open complex formation by *Eco* RNAP holoenzyme (20).

Previous studies demonstrated that the main role in DNA melting during open complex formation is played by region 2 of the σ subunit, with conserved aromatic and positively charged amino acids from this region directly involved in interactions with the −10 promoter element (Fig. 1*A*) (12–17, 59). Substitutions of nonconserved amino acid residues in region 2 were demonstrated to modulate the temperature of promoter opening and to be in part responsible for the cold sensitivity of DNA melting by *Taq* RNAP in comparison with *Eco* RNAP (38). Our results suggest that nonconserved amino acid substitutions in regions 1.2 and 2 can together determine the natural variations in promoter DNA melting in various bacteria.

The instability of promoter complexes formed by *Taq* RNAP is paralleled by a higher promoter escape efficiency displayed by this RNAP in comparison with *Eco* RNAP. We propose that these differences in part depend on the properties of the *Taq* σ^{A} subunit that was shown to stimulate promoter escape in comparison with the σ^{70} subunit when combined with *Eco* core RNAP. However, the higher promoter escape efficiency displayed by the σ^{A}-containing RNAP does not depend on σ regions 1.1 or 1.2 but is apparently explained by structural features of the C-terminal part of the subunit, including regions 2, 3, and 4. In particular, nonconserved amino acid substitutions in region 2 may likely affect promoter escape through changes in interactions of the σ subunit with the −10 promoter element and/or with core RNAP (38), whereas substitutions in regions 3 and 4 may change σ contacts with the growing RNA transcript during initiation (5, 6).

Although analysis of hybrid RNAPs utilized in this work do have some caveats, several lines of evidence support the validity of this approach for comparison of functional properties of σ subunits from different bacteria. First, the levels of promoter-dependent activities at the temperature optima were similar for the wild-type *Eco* and hybrid RNAPs containing *Taq* σ^{A} and mosaic σ subunits. Second, *Taq* σ^{A} in complex with *Eco* core RNAP efficiently recognized the −10 promoter element in the nontemplate promoter strand, suggesting that the σ subunit adopts a proper conformation for DNA binding. Third, many properties of the hybrid RNAPs expectedly reproduced the properties of *Taq* holoenzyme RNAP. Finally, individual substitutions of σ regions 1.1 and 1.2 replaced relatively small parts of the σ subunit and affected a subset of promoter complex properties, thus implying specific functions for these regions in transcription initiation.

In conclusion, our results suggest that regions 1.1 and 1.2 of the *Eco* σ^{70} and *Taq* σ^{A} subunits modulate conformational transitions of RNAP during open complex formation and determine significant differences between the *Eco* and *Taq* RNAPs in transcription initiation. Although the functional importance of these differences for the expression of bacterial genes remains to be established, it can be proposed that the observed evolutionary variations in the properties of the σ subunit may have an important role in transcription regulation.

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