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Region 1.2 of the RNA polymerase σ subunit controls recognition of the −10 promoter element

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Recognition of the −10 promoter consensus element by region 2 of the bacterial RNA polymerase σ subunit is a key step in transcription initiation. σ also functions as an elongation factor, inducing transcription pausing by interacting with transcribed DNA non-template strand sequences that are similar to the −10 element sequence. Here, we show that the region 1.2 of *Escherichia coli* σ^{70}, whose function was heretofore unknown, is strictly required for efficient recognition of the non-template strand of −10-like pause-inducing DNA sequence by σ region 2, and for σ-dependent promoter-proximal pausing. Recognition of the fork-junction promoter DNA by RNA polymerase holoenzyme also requires σ region 1.2 and thus resembles the pause-inducing sequence recognition. Our results, together with available structural data, support a model where σ region 1.2 acts as a core RNA polymerase-dependent allosteric switch that modulates non-template DNA strand recognition by σ region 2 during transcription initiation and elongation.

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Introduction

Initiation of transcription by RNA polymerase (RNAP) requires local melting of DNA around the transcription start site. In bacterial cells, a single auxiliary factor, the RNAP σ subunit, directs promoter recognition and opening (Gross *et al*, 1998; Helmann and deHaseth, 1999). In *Escherichia coli*, the σ^{70} subunit is responsible for transcription initiation from most promoters during exponential growth. During transcription initiation, the conserved regions 2 and 4 of σ^{70} recognize the −10 and −35 promoter elements, respectively. Specific recognition of the non-template strand of the −10 promoter element (consensus sequence 5′-$T^{-12}A^{-11}T^{-10}A^{-9}A^{-8}T^{-7}$-3′) by σ^{70} is required for localized melting of promoter DNA (Juang and Helmann, 1994; Roberts and Roberts, 1996; Marr and Roberts, 1997; Panaghie *et al*, 2000; Young *et al*, 2001). However, free σ^{70} does not bind promoter DNA. A coiled-coil domain of the RNAP β′ subunit induces a conformational change in σ^{70} that allows DNA binding and −10 element recognition by σ region 2 (Callaci *et al*, 1999; Kulbachinskiy *et al*, 1999; Young *et al*, 2001). Detailed mechanism of this conformational change is not known.

Recent studies have shown that σ^{70} can be retained in early transcription elongation complexes (TECs) *in vitro* (Bar-Nahum and Nudler 2001; Kapanidis *et al*, 2005) and *in vivo* (Raffaelle *et al*, 2005). This retention occurs when RNAP transcribes through a promoter-proximal sequence resembling the −10 promoter element. In these conditions, σ^{70} 'hops' from its initial promoter location and binds the non-template strand of the −10-like sequence, inducing a promoter-proximal pause (Ring *et al*, 1996; Brodolin *et al*, 2004; Nickels *et al*, 2004). The phenomenon of σ^{70}-dependent promoter-proximal pausing reveals that single-stranded DNA binding activity of σ^{70} region 2, which is strictly essential for promoter DNA recognition and melting, may hinder promoter escape and/or transcription elongation and therefore needs to be suppressed after the open promoter complex has been formed (Chan and Gross, 2001). In support of this idea, crosslinking experiments demonstrate that some σ^{70} interactions with the −10 promoter element are lost after full-length transcription bubble in the open promoter complex is established (Buckle *et al*, 1999; Brodolin *et al*, 2005).

To uncover the mechanism that regulates recognition of the non-template strand of the −10 element during transcription initiation and pausing, we developed a 'σ-exchange' assay that allows to exchange σ^{70} bound to paused elongation complexes with σ^{70} mutants. This assay bypasses the promoter recognition/opening steps and therefore allows one to study σ–TEC interactions even for σ derivatives that are inactive in transcription initiation. We analyzed interactions of different σ^{70} fragments with paused TEC and found that σ^{70} region 1.2 enhances intrinsic sequence-specific affinity of σ^{70} region 2 to single-stranded −10 promoter element DNA in the context of TEC. We propose that modulation of σ region 2 interactions with single-stranded DNA by region 1.2 is part of a regulatory mechanism that operates during transcription initiation and elongation. This mechanism allows RNAP to relinquish initial strong interactions with the −10 element DNA and escape into productive elongation.

Results

σ^{70} region 2 crosslinks to guanine at position +5 of promoter-proximal pause-inducing sequence

We used formaldehyde crosslinking to identify close σ^{70}-DNA contacts in the transcription bubble of TEC paused at the

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*lac*UV5 promoter positions +16/17. Previously, we localized a σ^{70} crosslink in paused TEC16/17 to between positions +4 and +6 of the non-template DNA strand (Brodolin *et al*, 2004). Substitution of the non-template guanine at position +5 to adenine (Figure 1A) has no effect on promoter-proximal pause (Figure 1B), but selectively abolishes σ^{70}-DNA crosslink (the β′-DNA crosslink is unaffected by this substitution) (Figure 1C). Thus, G^{+5} crosslinks to σ^{70} in TEC16/17 and is also the only base that is crosslinked to σ^{70} by formaldehyde in TEC16/17.

The site of σ^{70} that crosslinks to G^{+5} was mapped using single-hit Met-specific chemical cleavage of σ^{70} crosslinked to radioactively labeled DNA (Grachev *et al*, 1989; Brodolin *et al*, 2000). In a control reaction, σ^{70} subunit affinity-labeled between the Met^{508} and Met^{561} by ^{32}P-labeled ribonucleotide (Severinov *et al*, 1994) was used. Incubation with the cleaving reagent CNBr resulted in the appearance of a number of radioactive bands whose mobility on SDS–PAGE was higher than that of the starting crosslinked material (Figure 1D and E). These bands correspond to either N- or C-terminal products of Met-specific cleavage of the crosslinked σ^{70} subunit. In the case of the control (Figure 1D, panel 'σ_m'), radioactive cleavage products corresponded to C-terminal cleavage products and the shortest labeled peptides resulting from cleavage at $Met^{456-489}$ were clearly visible (labeled 456–489C in Figure 1D). Cleavage of the crosslinked σ-DNA complex (Figure 1D, panel 'σ-DNA'; Supplementary Figure S1) resulted in a similar pattern of bands arising from cleavages in the central part of the σ^{70} (Met^{297}–Met^{379}), but bands corresponding to C-terminal cleavage products at $Met^{456-489}$ were not observed. Instead, a strong band, whose mobility is consistent with that expected for N-terminal product of cleavage at $Met^{456-489}$ (labeled 456–489N in Figure 1D), was observed. This indicates that the site of crosslinking to DNA is located N-terminal to $Met^{456-489}$ but C-terminal to $Met^{365,379}$, as bands corresponding to C-terminal cleavage products at these positions were present in both 'σ-DNA' and control 'σ_m' reactions. The region of σ^{70} between $Met^{365,379}$ and $Met^{456-489}$ includes the entire conserved region 2 of σ^{70} known to be involved in specific interaction with the −10 element DNA.

To additionally confirm the mapping result, we used partial degradation of crosslinked σ^{70}-DNA complexes with Cys- and Trp-specific chemical proteases (Supplementary Figure S1). The results were consistent with inferences made from Met-specific degradation, and indicated that a residue(s) of σ^{70} region 2 is within a 2-Å distance from G^{+5} of the pause-inducing sequence, and that region 2 is the only σ^{70} region that crosslinks to the non-template DNA strand in TEC16/17.

σ-exchange assay

As promoter-proximal pausing involves 'hopping' of σ from the promoter to the pause-inducing site, σ^{70} must transiently relinquish its contacts with DNA at the promoter and then establish new contacts at the pause site. At least some of the contacts with the core should also be broken and then re-established during this process. The σ-exchange assay relies on the ability of exogenously added σ to bind to σ-less TEC stalled in the vicinity of promoter-proximal pause (Figure 2A). As shown previously (Brodolin *et al*, 2004), ~50% of transcription complexes stalled at position +16 of the *lac*UV5 promoter by CTP deprivation retained

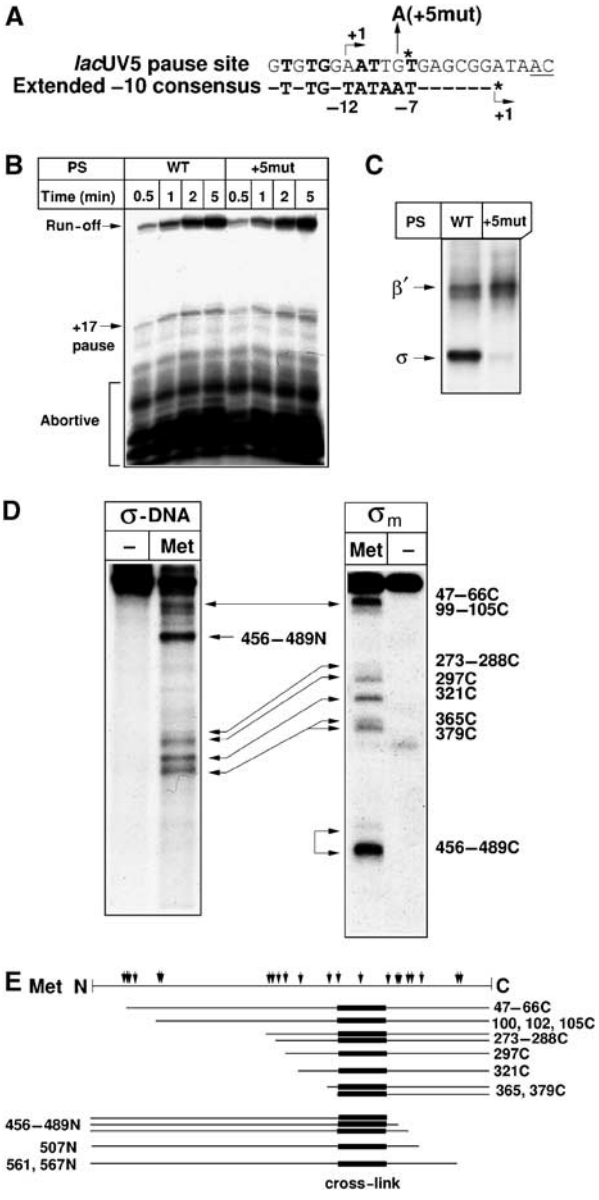

Figure 1 Mapping of the σ-DNA crosslink in paused TEC. (**A**) The *lac*UV5 pause-inducing sequence and the −10 promoter element consensus sequence (Keilty and Rosenberg, 1987). Matches to consensus are indicated in bold typeface. The +5G to A substitution is indicated (+5mut). An asterisk marks a position (+6T), which when substituted to A abolishes pausing (Brodolin *et al*, 2004). Transcription start sites (+1) are shown by arrows. (**B**) An autoradiogram of a denaturing gel showing ^{32}P-labeled RNA products produced during run-off transcription from the *lac*UV5 promoter fragments containing either WT or mutant (+5mut) pause-inducing sequence (PS). (**C**) Formaldehyde crosslinking of the +17 paused complexes formed at the WT or mutant (+5mut) ^{32}P-labeled *lac*UV5 promoter DNA. Crosslinked σ^{70}-DNA and β′-DNA complexes are labeled as σ and β′. (**D**) Mapping of the crosslinking site in the σ^{70} subunit crosslinked to ^{32}P-labeled *lac*UV5 DNA in TEC16 by Met-specific chemical cleavage (σ-DNA). The panel on the right (σ_m) shows Met-specific cleavage of the σ^{70} subunit ^{32}P-labeled at the C terminus. Continuous lines with arrows between panels σ_m and σ-DNA connect bands corresponding to the identical cleavage products. Positions of σ^{70} Met residues, which when cleaved give rise to observed cleavage products, are indicated on the right side of the figure. Suffixes C or N refer to C- or N-terminal cleavage product, respectively. (**E**) The diagram shows the map of the σ^{70} subunit; the positions of Met cleavage sites are indicated by arrows. ^{32}P-labeled peptides produced by cleavage at indicated σ^{70} Met residues are shown beneath as lines. σ^{70} region containing the crosslink site to DNA is indicated by a black bar.

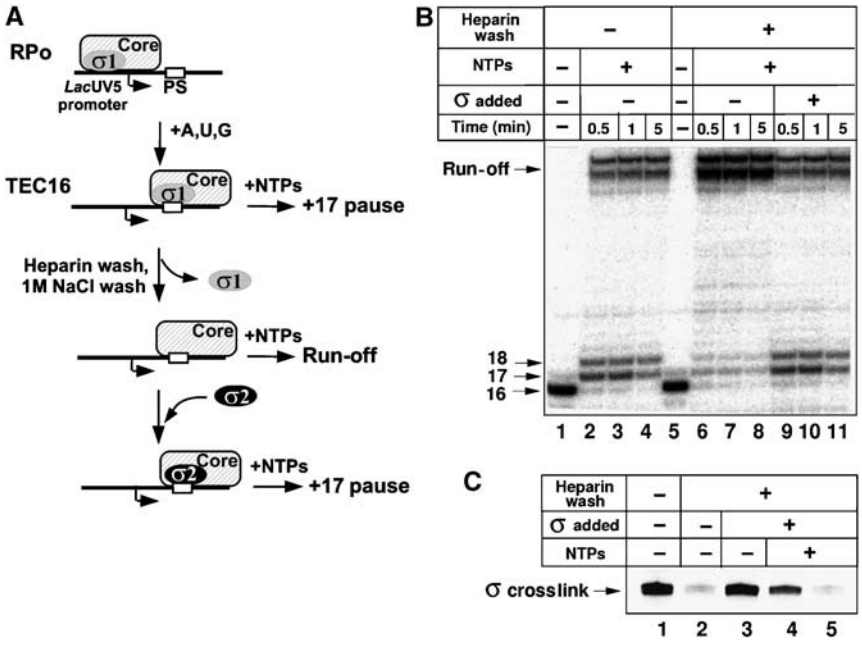

Figure 2 σ-exchange assay using immobilized +16 paused complexes. (**A**) A scheme demonstrating the principle of 'σ-exchange' experiments on the *lac*UV5 promoter-proximal pause. The pausing site (PS) is marked by an open rectangle. Promoter open complex (RPo) and paused elongation complex (TEC16) are shown. An exchange reaction between σ originated from the promoter complex (σ1 in gray) and exogenously added σ (σ2 in black) is illustrated. (**B**) ^{32}P-labeled RNA transcripts produced by σ^{70}-containing (lanes 1–4 and 9–11) and σ^{70}-less (lanes 5–8) TEC16 immobilized on Ni^{2+}-NTA beads. Transcription complexes were chased by the addition of NTPs and 0.5, 1, or 5-min incubation either before (lanes 2–4) or after heparin wash (lanes 6–11). Lanes 9–11: σ^{70}-less complexes were supplemented with exogenous σ^{70} (500 nM final concentration) before chase. (**C**) Crosslinking of the σ^{70} subunit to ^{32}P-labeled *lac*UV5 DNA in immobilized TEC16 either before (lane 1) or after (lanes 2–5) heparin wash. Complexes were supplemented with exogenous σ^{70} (lanes 3–5). NTPs were added before (lane 5) and after (lane 4) the addition of σ^{70}. Crosslinked σ^{70}–DNA complexes resolved on SDS–PAGE are shown (labeled as σ-crosslink).

σ and, upon the addition of full complement of NTPs, paused at position +17 owing to σ^{70} interactions with the pause-inducing sequence (Figure 2B, lanes 1–4). An additional pause at +18 was also observed, as reported previously (Nickels *et al*, 2004). Upon σ^{70} removal by treating the initial TEC16 with heparin, the +17/+18 pause disappeared and the full-sized run-off transcript was observed upon the addition of NTPs (Figure 2B, lanes 5–8). Importantly, the +17 pause was restored upon addition of σ^{70} (Figure 2B, compare lanes 2–4 and 9–11), indicating that exogenous σ^{70} binds stalled TEC and induces a promoter-proximal pause, presumably through interactions with the −10 element-like sequence.

Crosslinking experiments showed that incubation of heparin-washed TECs with excess σ^{70} for 5 min at 37°C resulted in the appearance of σ-DNA crosslinks (Brodolin *et al*, 2004, Figure 2C, lane 3). The σ^{70}-DNA crosslink was also observed when σ^{70}-less TEC16 supplemented with σ^{70} was incubated with NTPs for 2 min before crosslinking (Figure 2C, lane 4). The result is consistent with the results of transcription experiment that showed that a promoter-proximal pause is formed at these conditions (Figure 2B, lanes 9–11). In contrast, no crosslink was observed when NTPs were added before the addition of σ^{70} (Figure 2C, lane 5), further supporting an idea that σ^{70}-DNA crosslink in TEC16/17 depends on σ^{70} interactions with the pause-inducing sequence (see also above). We therefore conclude that TEC16 recruits exogenously added σ^{70}, and that the newly bound σ^{70} interacts with DNA and blocks RNAP escape into productive elongation. These results strongly suggest that externally added σ^{70} interacts with the pause-inducing sequence and RNAP core in the same way as 'endogenous' σ^{70} that originates from a promoter complex and causes promoter-proximal pausing. These observations form the basis of a 'σ-exchange' assay, in which different σ^{70} mutants are tested for their ability to bind σ-less TEC16 and induce promoter-proximal pausing.

Region 1.2 of σ^{70} is essential for −10-like pause-inducing sequence recognition

To determine σ^{70} regions that are essential for interaction with TEC16/17, a series of recombinant σ^{70} fragments was prepared (Figure 3A). The boundaries of these fragments were selected such that structural domains of σ^{70}, as defined by biochemical and crystallographic studies (Malhotra *et al*, 1996; Severinova *et al*, 1996; Campbell *et al*, 2002), remained intact. The σ^{70} fragments used lacked the N-terminal region 1.1 and parts of region 1.2 (σ_{2-4}, amino acids 102–613) or C-terminal regions 3 and 4 (σ_{1-2}, amino acids 1–448). In addition, a fragment missing both N-terminal regions and C-terminal region 4.2 (σ_{2-3}, amino acids 102–574) was created. The shortest fragment used in our experiments, σ_{2a} (amino acids 102–448), corresponds to the previously characterized fragment of σ^{70}, whose structure has been solved and that binds the RNAP core, forming a complex that recognizes the −10 promoter element in a single-stranded form (Malhotra *et al*, 1996; Severinova *et al*, 1996; Marr and Roberts, 1997). As deletion of N-terminal 101 amino acids of σ^{70} disrupts region 1.2, we also prepared an additional fragment σ_{2b} (amino acids 94–448) that has this region intact. None of the σ fragments studied was capable of directing promoter-dependent transcription by the core (data not shown).

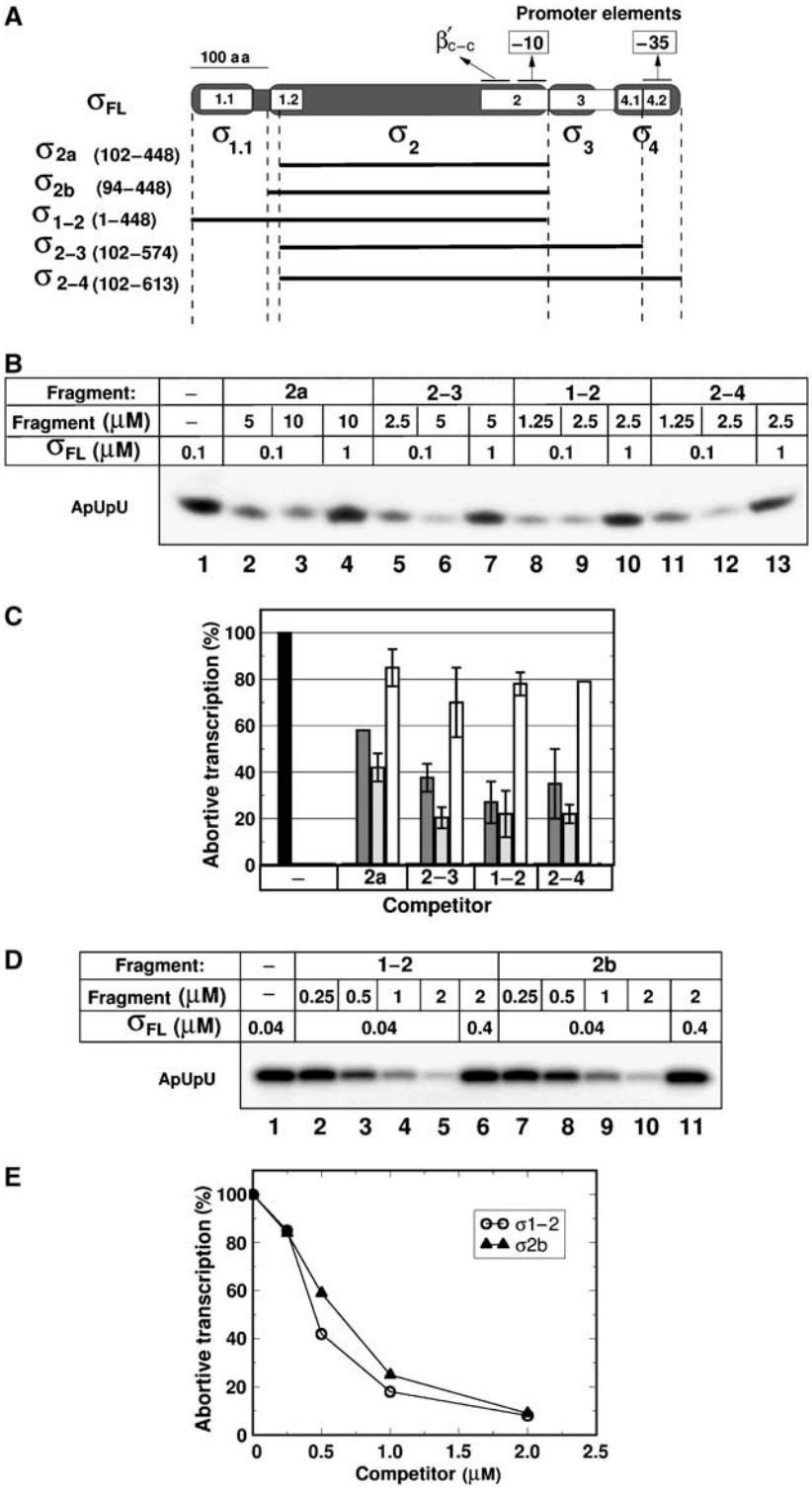

Figure 3 σ^{70} fragments used and their binding to RNAP core. (**A**) A scheme of σ^{70}; the universally conserved regions are shown in white and are numbered. The structural domains of σ (σ1.1, σ2, σ3, and σ4; Campbell *et al*, 2002) are indicated beneath. σ fragments used in this work are shown as simple lines below the σ^{70} scheme. Contact sites with the −10 and −35 promoter elements and the β′ coiled-coil (β′c–c) are indicated. (**B, D**) Inhibition of σ^{70}-dependent abortive transcription initiation reaction from the *lac*UV5 promoter by σ^{70} fragments. Concentrations of σ^{70} and its fragments are indicated at the top of the panel. Reactions contained either 50 nM (panel B) or 10 nM (panel D) of RNAP core. ^{32}P-labeled abortive ApUpU RNA product is marked. (**C**) Quantification of experimental data presented in panel B. The amount of ^{32}P-labeled RNA synthesized in the presence of σ fragments was normalized to the amount synthesized without fragments added (black bar at the left). Gray bars correspond to lanes 2, 5, 8, and 11 of panel B. Light gray bars correspond to lanes 3, 6, 9, and 12. White bars correspond to lanes 4, 7, 10, and 13. Mean values and s.d. from two independent experiments are shown. (**E**) Quantification of experimental data presented in panel D. The amount of ^{32}P-labeled abortive product synthesized in the presence of indicated concentrations of σ fragments is shown.

The σ-exchange assay is based on σ ability to bind core RNAP in the context of transcription elongation complex. Thus, for every σ fragment, it was essential to determine a concentration range that will allow efficient binding to the core. The relative affinities of σ fragments to RNAP core were measured by determining their ability to competitively inhibit σ^{70}-dependent abortive initiation (Severinova *et al*, 1996; Sharp *et al*, 1999), as well as by the native gel analysis, where the formation of RNAP holoenzyme from the core and σ or its fragments is monitored directly (Figure 3 and Supplementary Figure S2). Fragments σ_{1-2}, σ_{2-4}, and σ_{2b} efficiently bound the core, whereas σ_{2a} and σ_{2-3} bound poorly (Figure 3B and D). In agreement with previous results (Severinova *et al*, 1996), a 50-fold molar excess of σ_{2a} and σ_{2-3} over wild-type σ^{70} inhibited transcription by about 50–70% (Figure 3B, lanes 2, 3, 5, 6 and panel C). The addition of increased amounts of σ^{70} overcame the inhibition (Figure 3B, lanes 4 and 7), indicating that these fragments retained the binding specificity but lost their affinity. The results also indicate that region 1.1 does not contribute to the strength of σ^{70}–core interactions, as σ_{1-2} and σ_{2b} bind core with similar affinity (Figure 3D and E). On the other hand, the poor binding of σ_{2a} shows that region 1.2 amino acids 94–101 are important for efficient binding to the core. However, the presence of region 4 in the σ_{2-4} fragment allows efficient core binding even in the absence of these amino acids (Figure 3B, compare lanes 8, 9 and 11, 12). Thus, both regions 1.2 and 4 of σ appear to independently contribute to the interaction with the RNAP core.

To define regions of σ^{70} required for pause-inducing sequence recognition, we used the fragments described above in the σ-exchange assay. The exchange was followed by the appearance of crosslinks between σ fragments and promoter DNA. The concentrations of σ fragments were adjusted to allow equal efficiency of complex formation with the RNAP core. As can be seen, σ_{1-2} fragment was crosslinked to pause-inducing sequence as efficiently as σ^{70}, whereas fragments σ_{2a}, σ_{2-3}, and σ_{2-4} were not crosslinked (Figure 4A). Therefore, the N-terminal 101 amino acids of σ^{70}, which include the entire region 1.1 and a portion of region 1.2, are necessary for recognition of the pause-inducing sequence, whereas the C-terminal regions 3 and 4 are dispensable. In contrast to σ_{2a}, the σ_{2b} fragment (lacking 93 N-terminal amino acids, which constitute the entire region 1.1, but having intact region 1.2) recognized the pause site as efficiently as σ_{1-2} (Figure 4B, compare lanes 3 and 4). Efficient crosslinking was also observed when this fragment was added to σ-less TEC16 complexes before the addition of NTPs (Figure 4B, lanes 5 and 6), whereas no crosslinking was observed when NTPs were added before σ_{2b} (Figure 4B, lanes 8 and 9). Therefore, σ_{2b} blocked RNAP escape from the pause site with the same efficiency as σ_{1-2}. Thus, region 1.2 amino acids 94–101 are essential for formation of a contact between σ region 2 and the −10-like pause-inducing sequence and formation of a promoter-proximal pause.

To directly show that σ^{70} residues 94–101 are required for promoter-proximal pausing, we compared the ability of σ_{1-2}, σ_{2a}, σ_{2b}, and σ_{2-4} fragments to induce pausing at the +17

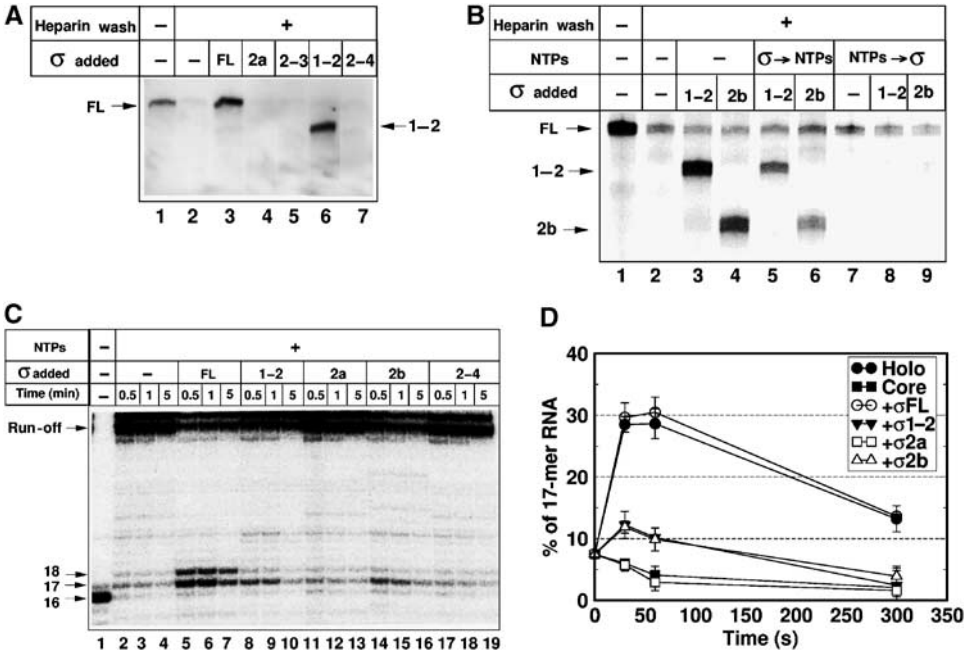

Figure 4 Binding of the exogenously added σ^{70} fragments to paused TEC16. (**A**) Crosslinking of TEC16 complexes to ^{32}P-labeled *lac*UV5 DNA before (lane 1) and after (lanes 2–7) heparin wash. Heparin-washed σ^{70}-less complexes were supplemented with 0.5 μM of wild-type σ^{70} (FL) or fragments: 5 μM of σ_{2a}, 3 μM of σ_{2-3}, 1.5 μM of σ_{1-2}, and 2.5 μM of $\sigma_{2.4}$ (lanes 3–7). Crosslinked complexes were resolved by SDS–PAGE and revealed by autoradiography. Crosslinked subunits are indicated. (**B**) Crosslinking of TEC16 complexes washed with heparin (lanes 2–9) and supplemented with 1.5 μM of σ_{2b} or σ_{1-2} (lanes 3–6, 8, and 9). NTPs were added before (lanes 7–9) or after (lanes 5 and 6) the addition of σ^{70} fragments. Lane 1: TEC16 before heparin wash. Reaction products were analyzed as in panel A. (**C**) ^{32}P-labeled RNA transcripts produced upon addition of NTPs to immobilized TEC16. Complexes were chased by the addition of NTPs for 0.5, 1, and 5 min. Lanes 5–19: complexes were supplemented with wild-type σ^{70} (FL) or indicated σ^{70} fragments before addition of NTPs. (**D**) Quantification of the results of experiment shown in panel C. The amount of +17 RNA was calculated as percentage of the initial amount of the starting 16-mer RNA present before the addition of NTPs. Mean values and s.d. from two independent experiments are shown.

position (Figure 4C). Heparin-washed TEC16 were incubated with σ^{70} fragments, supplemented with NTPs, and RNA products were analyzed at different time points after NTP addition (0.5, 1, and 5 min). As can be seen, σ_{1-2} and σ_{2b} induced a pause at +17 (Figure 4C, lanes 8–10 and 14–16), whereas fragments lacking the first 101 amino acids did not (Figure 4C, lanes 11–13 and 17–19). Noticeably, the +18 pause that was observed upon addition of σ^{70} was not observed with the fragments (Figure 4C, compare lanes 5–7 and 8–10). The reason(s) for this difference was not further investigated. Quantification revealed that in the case of σ^{70} fragments, a fraction of TEC16 retained in the +17 pause during the first 60 s of the chase was ~30% of that observed with full-length σ^{70} (Figure 4D). This lower efficiency of pausing is likely caused by lower affinity of σ^{70}_{1-2} and σ^{70}_{2b} for the elongation complex, either RNAP core or DNA (or both). In summary, we conclude that σ^{70} region 1.2 amino acids 94–101 are required for promoter-proximal pausing, whereas σ^{70} region 1.1 (amino acids 1–93) is dispensable.

Disruption of region 1.2 abolishes a conformational switch in σ^{70}

Specific recognition of the non-template DNA strand of the −10 promoter element is accomplished by residues of σ region 2 and requires a conformational transition in σ induced by the β′ subunit coiled-coil (Callaci and Heyduk 1998; Young *et al*, 2001). A plausible hypothesis is that deletion of region 1.2 residues 94–101 abolishes such a transition and therefore makes specific binding to single-stranded −10 element DNA impossible. To test this model, we performed UV crosslinking of RNAP holoenzymes containing σ^{70} or σ fragments with an oligonucleotide corresponding to the non-template strand of the −10 promoter element (−10 oligo) of the *lac*UV5 promoter. σ^{70} effectively crosslinks to the −10 oligo in the context of the holoenzyme, whereas isolated σ^{70} crosslinks weakly (Marr and Roberts, 1997; Kulbachinskiy *et al*, 1999; Young *et al*, 2001). The 'activation effect' of RNAP core on the interaction of σ^{70} or its fragments with the −10 oligo was measured as a ratio of the amount of DNA crosslinked to σ^{70} or its fragments in the presence or absence of the core (Figure 5A and B). At concentrations less than 500 nM, σ_{1-2} and σ_{2b} exhibited a ~10-fold activation effect comparable with that observed with σ^{70}, whereas only two-fold activation by σ_{2a} was observed, indicating that region 1.2 is required for −10 oligo recognition in the context of the holoenzyme. At higher concentrations (>1.5 μM), efficient crosslinks between the −10 oligo and σ^{70} or its fragments were observed even in the absence of RNAP core. Quantitative analysis demonstrated that free σ_{2a} and σ_{1-2} bound the −10 oligo with similar affinities (Supplementary Figure S3), suggesting that N-terminal 101 amino acids do not affect region 2 affinity for single-stranded DNA in the context of free σ. The crosslinks resulted from sequence-specific interactions, as they were not observed with control oligonucleotide corresponding to the −10 element template strand (Figure 5C, lanes 5–12). Therefore, region 2 of σ^{70} specifically recognizes the non-template strand of the −10 promoter element in the absence of RNAP core, albeit with low efficiency, and this interaction does not depend on region 1.2. However, only when region 1.2 is present σ^{70} can undergo a core-dependent conformational change(s) that leads to increased affinity of region 2 for the −10 element single-stranded DNA.

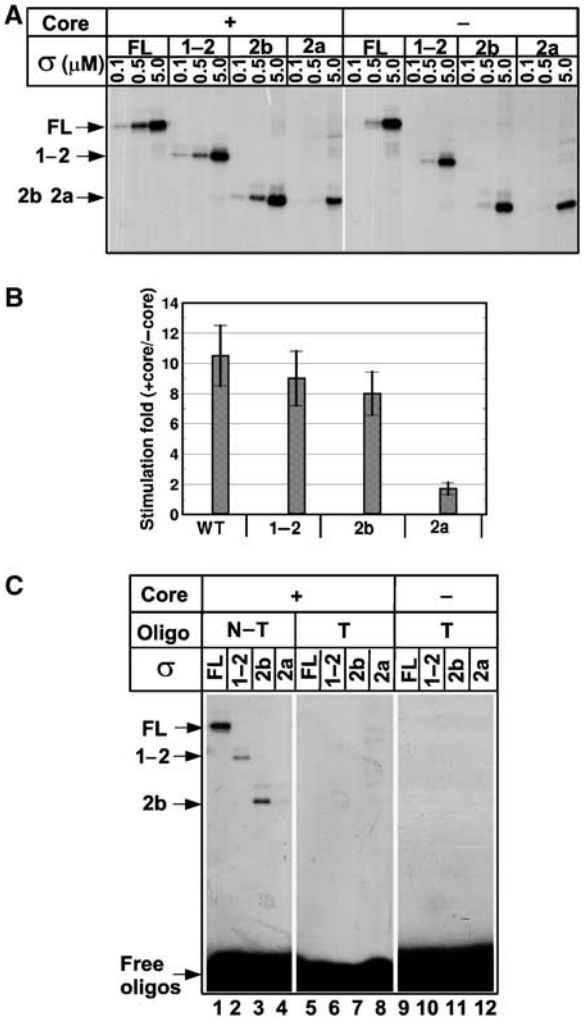

Figure 5 Probing of the interactions between σ^{70} or its fragments and the-10 oligonucleotide using UV crosslinking assay. (**A**) Crosslinking of the −10 non-template oligonucleotide with the wild-type σ^{70} (FL) or σ^{70} fragments (1–2, 2b, and 2a) present at the indicated concentrations in the absence or in the presence of 20 nM of RNAP core. Crosslinked complexes were resolved by 8% SDS–PAGE. (**B**) Quantification of data from panel A obtained in the presence of 0.1 μM σ^{70} or its fragments 'Stimulation fold' is a ratio of crosslinking signals observed in presence and in the absence of RNAP core. Mean values and standard deviations from three independent experiments are shown. (**C**) Crosslinking of the non-template (NT) (lanes 1–4) and control template (T) (lanes 5–12) −10 element oligonucleotides with RNAP holoenzymes (lanes 1–8) containing either wild-type σ^{70} (FL) or indicated σ^{70} fragments. Lanes 9–12: crosslinking of σ^{70} and σ^{70} fragments without core RNAP. The samples contained 0.5 μM (lanes 1–4) or 5 μM of σ (lanes 5–12).

Region 1.2 is required for fork-junction DNA binding

Two recently published studies suggested that σ^{70} region 1.2 interacts sequence specifically with the non-template DNA downstream of the −10 element in the context of open promoter complex (Feklistov *et al*, 2006; Haugen *et al*, 2006). Thus, deletion of σ^{70} residues 94–101 may abolish the −10 element recognition by disrupting this interaction. To explore this possibility, we used a fork-junction DNA template that mimic interactions of RNAP with upstream part of

the transcription bubble within the open promoter complex (Guo and Gralla, 1998).

The −10 promoter element bases −7 and −11, that are critical for formation of stable RNAP holoenzyme fork-junction DNA complexes (Guo and Gralla, 1998; Matlock and Heyduk, 2000; Fenton and Gralla 2001), correspond to promoter-proximal pause-inducing sequence bases +2 and +6 that are critical for pause formation (see Figure 1A; Ring *et al*, 1996; Brodolin *et al*, 2004). To increase a similarity between fork-junction DNA complex and complex paused at promoter-proximal pause, we used a fork-junction template that lacked a −35 promoter element sequence but contained an extended −10 element sequence corresponding to pause-inducing sequence of *lac*UV5 (Figure 6A). Formaldehyde crosslinking experiments revealed that fork-junction DNA efficiently crosslinked to σ^{70} in context of the holoenzyme, whereas no crosslinks were observed with RNAP core or free σ^{70} (Figure 6B, lanes 1 and 2 and data not shown). The crosslink was formed when a G residue was present at position −8 of the −10 element. Substitution of this G by C abolished the crosslink (Figure 6B, lane 3), but had no effect on RNAP holoenzyme affinity for the fork-junction DNA (data not shown). Taking into account that formaldehyde specifically targets unpaired guanines (Brodolin *et al*, 2000; K Brodolin, unpublished results), the result strongly suggests that G^{-8} is crosslinked to the σ^{70} in the fork-junction DNA–RNAP σ^{70} holoenzyme complex. Recall that a corresponding guanine at position +5 located in the pause-inducing sequence was also crosslinked to σ^{70} in TEC16 (Figure 1A and C). Therefore, the process of recognition of the −10 element in the context of fork-junction DNA complexes resembles the recognition of pause-inducing sequence in the context of

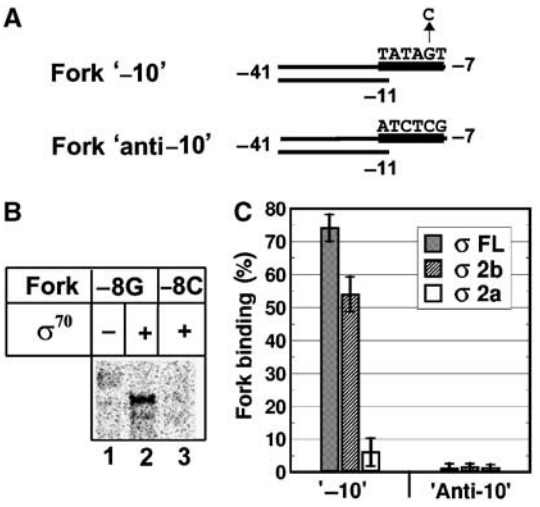

Figure 6 Interaction of fork-junction templates with holoenzymes containing the wild-type σ^{70} subunit or σ fragments. (**A**) A scheme of the fork-junction templates. The positions of −10 elements are marked by black rectangle and corresponding DNA sequences are shown. Arrow indicates substitution of the −8G to C. (**B**) Crosslinking of the core RNAP or σ^{70} containing holoenzyme to fork DNA. Templates contained at position −8 either G (−8G) or C (−8C). Crosslinked complexes were resolved by SDS–PAGE and revealed by autoradiography. (**C**) Complex formation between RNAP and forks bearing −10 element ('−10') or anticonsensus −10 sequence ('anti-10') was detected by nitrocellulose filtration method as described in Materials and methods. Binding is shown as percentage of total DNA in the sample. Mean values and s.d. from two independent experiments are shown.

TEC, and is therefore expected to be dependent on σ region 1.2. The requirement for region 1.2 should be independent of its recently proposed direct role in DNA binding (Feklistov *et al*, 2006; Haugen *et al*, 2006), as the non-template DNA strand of the fork-junction template ends at position −7 and hence does not contain region 1.2 binding site.

To explore the role of region 1.2 in fork-junction DNA binding, we analyzed interactions of RNAP holoenzymes containing fragments σ_{2a} or σ_{2b} with fork-junction DNA using a filter binding assay (Figure 6C). Preliminary experiments showed that recognition of fork-junction DNA by the σ^{70} holoenzyme strictly depended on the presence of the −10 element, as fork-junction templates containing the anticonsensus sequence instead of the −10 element sequence were not bound by RNAP (Figure 6C, 'anti-10'). Furthermore, holoenzymes containing σ^{70} or the σ_{2b} fragment efficiently bound fork-junction DNA, whereas binding by the holoenzyme containing σ_{2a} was much weaker (5–10% of that observed with σ^{70} or σ_{2b} holoenzymes, Figure 6C). Free σ subunit or its fragments did not bind fork-junction DNA, as expected. These results show that (1) RNAP binding to the −10 element in the context of fork DNA template is stimulated by region 1.2 and (2) the stimulation is independent of region 1.2 interactions with DNA downstream of the −10 element.

Discussion

Recognition of the −10 element non-template DNA

Our study of interactions between the bacterial RNAP σ^{70} subunit and transcription elongation complex revealed that σ^{70} region 1.2 amino acids 94–101 are required for specific interaction with the −10 promoter element (or −10-like pause-inducing sequence) non-template strand in the context of RNAP holoenzyme. On the other hand, region 1.2 is not required for specific, but low-affinity binding to single-stranded −10 promoter element DNA by free σ. Therefore, region 1.2 does not interact with the −10 element DNA by itself, but modulates the recognition of the −10 element by the σ subunit region 2 indirectly, that is, allosterically. We propose that region 1.2 stabilizes a conformation of region 2 that is required for optimal binding of the −10 element. One known allosteric effector that activates region 2 ability to bind and melt DNA is the β′ coiled-coil element (Young *et al*, 2001, 2004). We suggest that region 1.2 is also part of this activation mechanism that functions during transcription initiation and promoter-proximal pausing.

Our interpretation is supported by available structures of RNAP holoenzymes and promoter complexes. In Figure 7, a model of the transcription complex paused at a promoter-proximal site is presented. The model is based on the crystal structure of the *Thermus aquaticus* RNAP holoenzyme complexed with a fork-junction DNA template (Murakami *et al*, 2002a). The model takes into consideration the following data. First, our crosslink mapping data show that σ region 2 contacts the non-template G^{+5} in paused TEC16. Second, we assume, based on mutagenesis and biochemical data, that RNAP complex with fork-junction DNA mimic the σ-dependent interactions of RNAP with the pause-inducing sequence. These considerations suggest that the DNA melting region 2.3 of σ that contacts the position −8 of the fork-junction DNA also contacts (and crosslinks to) the +5 position of the

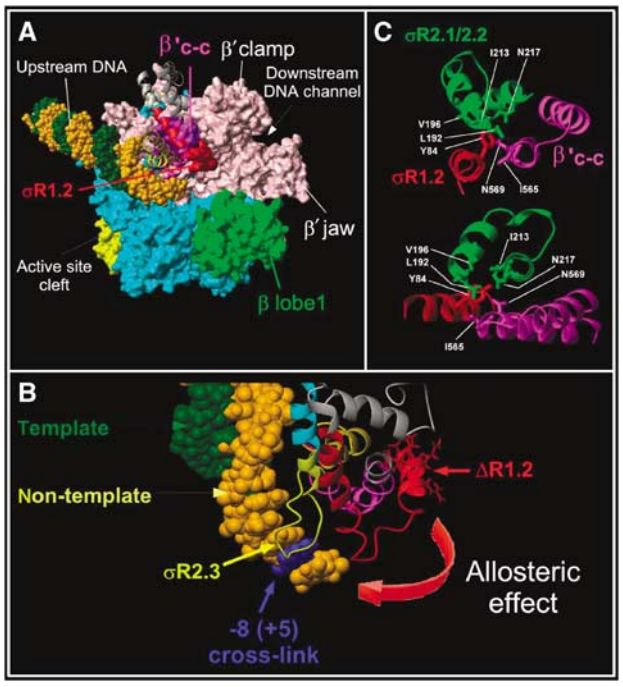

Figure 7 Modeling of the σ_{2b} fragment interactions with RNAP core on the crystallographic structures of *T. aquaticus* and *T. thermophilus* RNAP. (**A**) The structure of *T. thermophilus* RNAP (PDB accession number 1IW7) fitted with the *T. aquaticus* RNAP structure in the complex with fork-junction DNA (PDB accession number 1L9Z). RNAP is shown as a molecular surface and DNA is shown as a CPK structure with the non-template strand in orange and the template strand in green. The large non-conserved domain present in *Thermus* β′ is omitted for clarity. The σ_{2b} fragment is shown as ribbons with region 1.2 amino acids 94–101 shown as a red molecular surface. RNAP core subunits are colored as follows: β′ in rose and β in cyan; the β′ coiled-coil in violet. The β lobe that interacts with σ region 1.1 and forms the upper jaw is shown in green. (**B**) Modeling of σ crosslink in TEC16 on the *T. aquaticus* RNAP and fork-junction DNA complex structure. Color codes are as in panel A. The position corresponding to crosslinked +5G is shown in blue. (**C**) Interactions between σ region 1.2 (red) and 2.1–2.2 (green) and the β′ coiled-coil (magenta) in the *T. thermophilus* RNAP holoenzyme structure. Two projections are shown. The molecular modeling and figures were acquired using MOLMOL package (Koradi *et al*, 1996).

paused complex (Figures 6 and 7B). Our results show that formation of this contact is strictly dependent on region 1.2. According to the structure, there are no direct interactions between region 1.2 and the −10 element (Figure 7B). Furthermore, the fork-junction DNA complex is devoid of the non-template DNA strand downstream of the −10 element that might interact with region 1.2 in *E. coli* RNAP promoter complex (Haugen *et al*, 2006) or *T. aquaticus* σ^A–aptamer complex (Feklistov *et al*, 2006). Thus, a direct binding of region 1.2 to DNA is not required for core RNAP-dependent recognition of the −10 element.

As our data clearly indicate that region 1.2 is required for this recognition, region 1.2 may modulate the DNA-binding affinity of the region 2.3 through protein–protein interactions. Indeed, analysis of crystal structures of the σ^A RNAP holoenzymes from *Thermus thermophilus* and *T. aquaticus* (Vassylyev *et al*, 2002; Murakami *et al*, 2002a, b) reveals that region 1.2 amino acids 77–84 of *T. thermophilus* σ^A (*T. aquaticus* σ^A amino acids 92–99) that correspond to σ^{70} amino acids 94–101 form an α-helix (shown in red on Figure 7A) that contacts the β′ coiled-coil (shown in violet). In particular, conserved Tyr^{84} of *T. thermophilus* σ^A (*E. coli* σ^{70}-Tyr^{101}) interacts with the β′ coiled-coil Ile^{565} and Asn^{569} (*E. coli* Ile^{290} and Asn^{294}, correspondingly), σ^A region 2.1 Leu^{192} and Val^{196} (*E. coli* σ^{70} Leu^{384} and Val^{388}, correspondingly), and region 2.2 Ile^{213} and Asn^{217} (*E. coli* σ^{70} Ile^{405} and Asn^{409}, correspondingly) (Figure 7C). These residues of regions 2.1–2.2 also form a contact surface with conserved residues of the β′ coiled-coil. Thus, region 1.2 amino acids 94–101 interacts both with σ region 2 and the β′ coiled-coil and therefore may regulate the DNA binding activity of σ region 2.3.

The mechanism of activation of σ region 2 DNA binding by the β′ coiled-coil is not known. Even though the overall fold of σ domain 2 (regions 1.2 and 2) does not change significantly upon core binding (Campbell *et al*, 2002; Vassylyev *et al*, 2002; Murakami *et al*, 2002b), relative movement of α-helices of σ regions 2.1 and 2.2 is required for efficient promoter melting and open complex formation (Anthony and Burgess, 2002), and conformational changes within the 'DNA melting' region 2.3 were detected upon the holoenzyme formation (Callaci and Heyduk, 1998), suggesting that this region must adopt a specific conformation required for DNA binding. Region 1.2 is well positioned to act in concert with the β′ coiled-coil to lock region 2.3 in a conformation that is capable of strong interaction with the −10 element sequence. This interaction is required for initiation of promoter DNA melting at the −10 element (Guo and Gralla 1998; Fenton *et al*, 2000; Panaghie *et al*, 2000; Lim *et al*, 2001). In support of this idea, amino-acid substitutions in the region 1.2 blocked isomerization from closed to open promoter complex and promoter escape (Baldwin & Dombroski, 2001; Hsu *et al*, 2004).

Interestingly, our results show that free σ can specifically (but with low affinity) recognize non-template −10 oligo even without core RNAP. This finding contrasts with the results of previous studies showing that −10 oligo recognition is possible only in the RNAP holoenzyme complex (Marr and Roberts 1997; Kulbachinskiy *et al*, 1999; Young *et al*, 2001). We suggest that the reason for this difference is that interactions between σ and non-template −10 oligo have never been studied at the high concentrations of σ (1–5 μM) that we have used. Furthermore, the in-solution method of detection used by us, UV crosslinking, allows to detect weak interactions that could have escaped detection by non-equilibrium methods, that is, filer binding assay or gel shift, used previously.

Function of region 1.2 in pausing and promoter escape

We show that a minimal fragment of σ^{70} that is sufficient for binding to transcribing RNAP and causing promoter-proximal pause corresponds to the σ_2 structural domain (σ^{70} regions 1.2–2), which is conserved in all σ^{70} family proteins (Severinova *et al*, 1996, Campbell *et al*, 2002). Therefore, it appears that all σ^{70} family proteins may act as elongation factors, recognizing specific −10-like sequences within transcription units *in vivo*. Interestingly, pausing is induced by σ^{70} or σ^{70} fragments at concentrations (0.5–2 μM) that are below the concentration (~11 μM) estimated for σ^{70} *in vivo* (Mooney and Landick, 2003).

An important conclusion that follows from our results is that disruption of contacts between σ regions 3–4 and core RNAP does not prevent the −10 element recognition, and is therefore not sufficient to allow promoter escape. This

indicates that a different mechanism that disrupts σ interactions with the single-stranded promoter DNA at later stages of transcription initiation may exist. We speculate that region 1.2 is involved in this mechanism, activating/inactivating the −10 element binding during transcription initiation. As was noted by Vassylyev *et al* (2002), the α-helix of region 1.2 and the β subunit Lobe 1 block the entry of double-stranded downstream DNA into its binding site. Therefore, RNAP interactions with downstream DNA may influence the conformation/position of σ region 1.2. In addition, σ region 1.1 that undergoes a large-scale movement upon open promoter complex formation (Mekler *et al*, 2002) may physically pull the adjacent region 1.2, and thus influence its orientation relative to σ region 2 and the β′ coiled-coil. Disruption of contacts between region 1.2 and β′ caused by either of these processes may result in weakening of interactions between region 2.3 and the −10 element. Weakening the interactions with the −10 element creates a prerequisite for σ dissociation after the growing nascent RNA chain disrupts contacts with the core made by C-terminal domains of σ. The proposed mechanism of region 1.2-mediated −10 element binding is functioning in all experimental systems that require specific recognition of the −10 promoter element single-stranded DNA by σ region 2 (fork-junction DNA–RNAP holoenzyme complex, −10 oligonucleotide–RNAP holoenzyme complex, and elongation complex paused at a promoter-proximal pause site) and should operate for all σ^{70} family of transcription factors.

Materials and methods

Proteins and DNA

E. coli RNAP containing N-terminally His_6-tagged β′ was purified as described (Brodolin *et al*, 2000). The wild-type σ^{70} subunit was purified from an overexpression strain as described (Gribskov and Burgess, 1983). To construct σ^{70} deletions, the *rpoD* gene was cloned in the pET28 vector at *Nde*I–*Eco*RI sites. PCR mutagenesis was performed by amplifying the required fragments with primers containing the *Nde*I site at the N terminus and the *Eco*RI site at the C terminus and recloned at the same vector. Expression in *E.coli* BL21(DE3) strain and purification using chromatography on Ni^{2+}-NTA agarose column (Qiagen) of the σ fragments were performed as described (Wilson and Dombroski, 1997). All the fragments had His_6 tags at the N terminus that did not interfere with binding to the core (Severinova *et al*, 1996). The *lac*UV5 promoter fragments (−59 to +58), either wild type or containing a +5G to A substitution, were prepared by PCR, ^{32}P-labeled as described (Brodolin *et al*, 2004), and purified on 7% PAGE.

Transcription in vitro, core-binding assay and σ-exchange crosslinking assay

Transcription was performed in 10 μl of transcription buffer (TB) (40 mM HEPES pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 5% glycerol) containing 200 nM RNAP and 20 nM *lac*UV5 fragment incubated at 37°C for 10 min to form an open complex. Transcription was initiated by addition of the mixture of 0.5 mM ApA and 40 μM NTPs. In order to label RNA, 2 μCi of [α-^{32}P]UTP was added per reaction.

Core-binding competition assay was performed as follows. Core RNAP (10 nM or 50 nM) and σ^{70} or σ^{70} fragments in amounts indicated in the figures were incubated in 10 μl of TB for 10 min at 37°C. Then the DNA fragment (3 nM) containing the *lac*UV5 promoter, 0.5 mM ApA, and 40 μM [α-^{32}P]UTP were added and the samples were incubated for 5 min at 37°. RNA was analyzed on denaturing 24% PAGE.

Transcription complexes stalled at position +16 of the *lac*UV5 promoter were prepared and immobilized on Ni^{2+}-NTA agarose (Qiagen) via the His_6 tag, essentially as described (Brodolin *et al*, 2004). To remove σ^{70} subunit from the stalled complexes, they were washed with TB containing 200 μg/ml heparin for 5 min at 37°C. Heparin was removed by extensive washing with TB containing 1 M NaCl and then with TB. A 0.5 μM portion of σ^{70}, 5 μM of $\sigma_{102-448}$, 3 μM of $\sigma_{102-574}$, 1.5 μM of σ_{1-448}, or 2.5 μM of $\sigma_{102-613}$ or 1.5 μM of σ_{94-448} were added to σ^{70}-less complexes where indicated. NTPs were added to 100 μM before or after σ^{70} or σ^{70} fragments and the samples were incubated for 5 min at 37°C. Crosslinking was performed for 30 s with 30 mM formaldehyde (Brodolin *et al*, 2000). Crosslinked complexes were analyzed on 5% SDS–PAGE.

Limited chemical cleavage of crosslinked complexes

Cleavage at Met residues with CNBr and at Cys with TNCBA was performed as described (Brodolin *et al*, 2000). Cleavage at Trp with *N*-bromosuccinimide was performed as follows. Crosslinked complexes eluted from gel were dissolved in 0.5% SDS and pH was adjusted to 4.0 with the HCOOH-Na buffer. *N*-bromosuccinimide was added at 0.1 mM and the samples were incubated for 5 min at room temperature. Reaction was terminated by addition of equal volume of a Laemmli loading buffer and immediately loaded on SDS–PAGE.

UV light crosslinking of −10 oligonucleotides

Non-template and template oligonucleotides used in the crosslinking experiments were closely related to the *lac*UV5 promoter −10 element (Supplementary data) Labeling, purification, and crosslinking of the oligonucleotides was performed as described (Kulbachinskiy *et al* 1999). Crosslinked product were resolved on 8% SDS–PAGE.

Fork-junction DNA crosslinking and filter binding

Fork-junction DNA was prepared by annealing two oligonucleotides corresponding to the template and non-template DNA strands (Supplementary data). The template oligonucleotide was labeled at the 5′-end by [γ-^{32}P]ATP. The resulting fork-junction contained a 30 nt long double-stranded region followed by a 5-nt single-stranded extension of the non-template strand corresponding to the −10 promoter element. In control experiments, we used a fork-junction that contained C instead of G at the fifth position of the −10 element (TATACT) or the 'anticonsensus' sequence of the −10 element (ATCTCG). In the binding assays, core enzyme RNAP (3 nM) was incubated with either the full-length σ^{70} subunit (100 nM) or σ^{70} fragments 2a (5 μM) and 2b (2 μM) for 10 min in 50 μl of the binding buffer (20 mM Tris–HCl, pH 7.9, 240 mM NaCl, 60 mM KCl, and 10 mM $MgCl_2$) and fork-junction DNA was added at 3 nM. The incubation continued at 25°C for another 30 min. The samples were filtered through 0.45 μm nitrocellulose filters (HAWP, Millipore). The filters were washed with 5 ml of the buffer and quantified with PhosphorImager.

Formaldehyde crosslinking of RNAP holoenzyme (200 nM) in complex with fork-junction template (10 nM) was performed in TB containing 200 mM NaCl (Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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