Interaction with free β′ subunit unmasks DNA-binding domain of RNA polymerase σ subunit

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Abstract The promoter recognition site on the σ^{70} initiation factor is shielded from interaction with DNA unless σ^{70} is bound to the core component of RNA polymerase (RNAP). It is shown that interaction of σ^{70} with the isolated β′ subunit of *Escherichia coli* RNAP is sufficient to induce unshielding of the DNA binding site. Using UV-induced DNA-protein cross-linking we demonstrate that free β′ stimulates specific cross-links between region 2 of the σ^{70} polypeptide and a fragment of the non-template promoter strand containing the TATAAT sequence. Thus the σβ′ subassembly of RNAP can assume a functionally competent conformation independently of the bulk of the RNAP core.

Key words: RNA polymerase; Promoter recognition; UV cross-linking; *Escherichia coli*

1. Introduction

DNA-dependent RNA polymerase (RNAP) of *Escherichia coli* is a prototype of a large multisubunit cellular RNA polymerase that has been conserved throughout evolution [1]. Dissection of the multifunctional RNAP molecule into constituent structure-functional components is a prerequisite for understanding of transcription and its regulation in molecular detail.

Each of the two largest subunits of RNAP, β and β′, is composed of four structural modules, which are separated by stretches of non-essential amino acid sequences or can be genetically split from each other [2]. At least six different modules of β and β′ participate in the formation of the active center indicating that the two subunits are intimately interwound in the core globule [3]. To date no distinct function could be demonstrated for an isolated large subunit or its module.

The minimal functional subassembly of subunits is the 390 kDa core enzyme ($\alpha_2\beta\beta'$) which harbors the active center and can perform processive elongation of RNA chains. However, to bind to a promoter, a σ subunit is required. In *E. coli*, the major σ subunit (σ^{70}) carries promoter recognition sites in its C-proximal half [4,5]. In free σ^{70}, these sites are masked through interaction between the C-terminal and N-terminal domains but become exposed when σ binds to the core yielding the RNAP holoenzyme ($\alpha_2\beta\beta'\sigma$) [6].

σ^{70} was shown to interact with the isolated β′ but not β subunit which suggested that the major σ^{70} binding site of the core polymerase was located on β′ [7,8]. However, it remained unclear whether this β′σ interaction was functionally important, e.g. resulted in conformational changes of σ^{70}. In a reaction mimicking promoter binding, short fragments of the non-template strand carrying the '−10' TATAAT motif bind preferentially to σ^{70} within the holoenzyme [9–12]. Here we show that such specific binding to σ^{70} also occurs within the β′σ subassembly independently of the bulk of the core. Thus, direct interaction between free β′ and σ subunits can yield functionally competent tertiary structure with the promoter single-stranded (ss) DNA recognition site of σ exposed.

2. Materials and methods

2.1. Oligos

Oligonucleotides shown in Fig. 1A were purchased from Operon Technologies, Inc. 50–150 pmol of oligo were labeled with 50 μCi of [γ-^{32}P]ATP (NEN) with 25 units of T4 polynucleotide kinase (New England Biolabs) and purified by electrophoresis in non-denaturing 15% PAGE.

2.2. RNAP and subunits

E. coli core polymerase was purified according to [13] with modifications including FPLC Superose 6 gel filtration (10/30 HR, Pharmacia Biotech) followed by MonoQ 5/5 ion exchange chromatography [14]. *E. coli* σ^{70} was purified as described [14]. β (encoded by pMKSe2 [15]) and β′ (encoded by pT7β′ [16]) were overexpressed in *E. coli* XL1-blue and BL21(λDE3), respectively, dissolved in buffer containing 6 M guanidine chloride and renatured by dialysis against buffer without denaturing agent (for details see [14]), purified from aggregates by gel filtration (Superose 6 column), concentrated by ultrafiltration in a Centricon-100 device (Amicon), supplemented with glycerol to 50%, and stored at −20°C.

2.3. UV cross-linking

All experiments were done in transcription buffer containing 40 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 5% glycerol and 100 mM NaCl unless otherwise indicated. Typically, 1.5 pmol of core polymerase or isolated β or β′ (1–5 pmol) were incubated with σ^{70} (0.15–30 pmol) for 10 min at 37°C in 15 μl. Then an appropriate oligo was added (0.3 pmol in most experiments) and after incubation for 10 min at 20°C samples were irradiated for 10 min under a 254 nm UV lamp (4 W Spectroline) placed on the top of open Eppendorf tubes. For preparative cross-linking, we used either 15 pmol of core polymerase, or 15 pmol of core plus 30 pmol of σ^{70}, or 20 pmol of β′ plus 30 pmol of σ^{70} in 30 μl reaction mixture supplemented with 5 pmol of an oligo.

2.4. Mapping cross-linking sites

Subunits, cross-linked with oligos, were separated by 4.5% SDS-PAGE electrophoresis, eluted with 400 μl of 0.03% SDS, freeze-dried on a SpeedVac and then dissolved in 30 μl of 50 mM HCl [17]. 10 μl

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Abbreviations: RNAP, RNA polymerase; nt-oligo(s), oligonucleotide(s) corresponding to the non-template strand of the −10 promoter region; t-oligo(s), oligonucleotide(s) corresponding to the template strand of the −10 promoter region; ssDNA, single-stranded DNA

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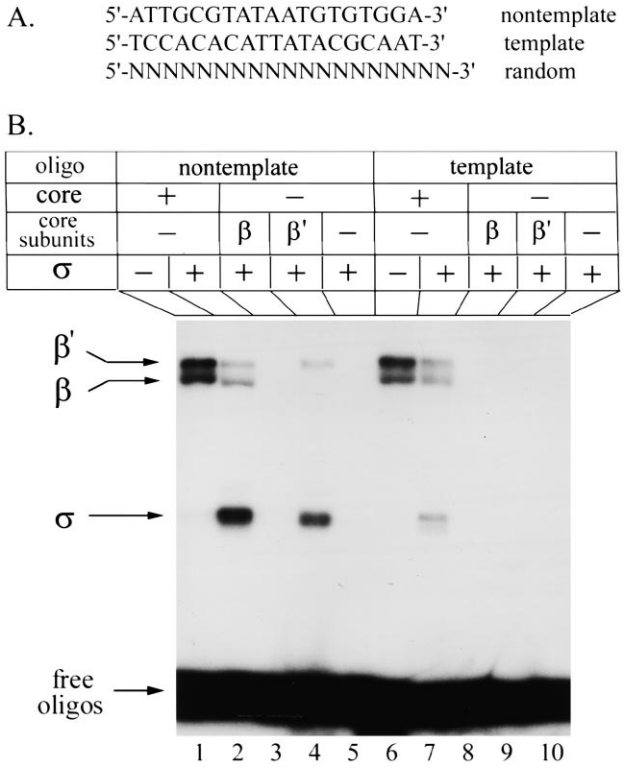

Fig. 1. Stimulation of σ^{70}-oligo cross-linking by isolated β′. A: The oligos used in the study. The nt-oligo is closely related to the non-template strand of the −10 region of the *lac*UV5 promoter, t-oligo is complementary to the nt-oligo, and random oligo is a statistic mixture of oligos of this size with all possible sequences. B: 1.5 pmol of core polymerase (lanes 2 and 7), 4 pmol of β (lanes 3 and 8) or β′ (lanes 4 and 9) were supplemented with 3.0 pmol of σ^{70} and incubated for 10 min at 37°C. Samples in lanes 1 and 6 contained only core (1.5 pmol) while samples in lanes 5 and 10 contained only σ^{70} (3.0 pmol). 0.3 pmol of nt- (lanes 1–5) or t- (lanes 6–10) oligo was added and after 10 min at 20°C samples were irradiated as described in Section 2. Radiolabeled subunits were separated on 4.5% SDS-PAGE and visualized by autoradiography.

aliquots were withdrawn and 1 M CNBr was added to the remaining samples to 50 mM concentration. The reaction was stopped after 5 and 10 min by adding an equal volume of a mixture containing 2% SDS, 0.5M Tris-HCl, pH 8.4, 100 mM β-mercaptoethanol and 20% glycerol. The degradation products were separated on 7–14% gradient SDS-PAGE.

3. Results

3.1. Cross-linking of specific nt-oligo to σ^{70} is induced by isolated β′ subunit

Oligonucleotide fragments of the non-template strand of the −10 promoter region (nt-oligos) can be specifically cross-linked to the σ^{70} subunit in the context of RNAP holoenzyme [10,11,18]. We confirmed this observation in the experiment of Fig. 1, using as the control the corresponding template strand oligonucleotide (t-oligo). It can be seen that free σ^{70} did not cross-link with either probe (lanes 5, 10) while core efficiently stimulated cross-linking of nt- but not t-oligo to σ^{70} (compare lanes 2 and 7).

The principal result of this experiment is the finding that isolated β′ subunit promoted cross-linking of σ^{70} with nt- but not t-oligo (lanes 4 and 9) while isolated β subunit had no such effect (lanes 3 and 8). At saturation with β′, stimulation of σ-nt-oligo crosslinking was 20–25% of that observed in the holoenzyme. Rigorous controls were performed to rule out the presence of core contamination in the β′ subunit preparations (data not shown).

To localize the cross-linking sites in the σ^{70} subunit we used the method of limited chemical cleavage of modified protein at Met residues [19]. The treatment of a protein, radiolabeled at a unique site, with BrCN under single-hit conditions results in the formation of two nested sets of radioactive peptides each containing the N- or C-terminus of the original protein. In most studied cases ligand binding changes RNAP subunit fragment gel mobility in a uniform way. This permits fragment identification by comparison of a theoretical pattern of cleavage of the non-modified subunit (Fig. 2A) with the experimental pattern of labeled fragments (Fig. 2B) rather than by measuring absolute masses of the fragments. The identifi-

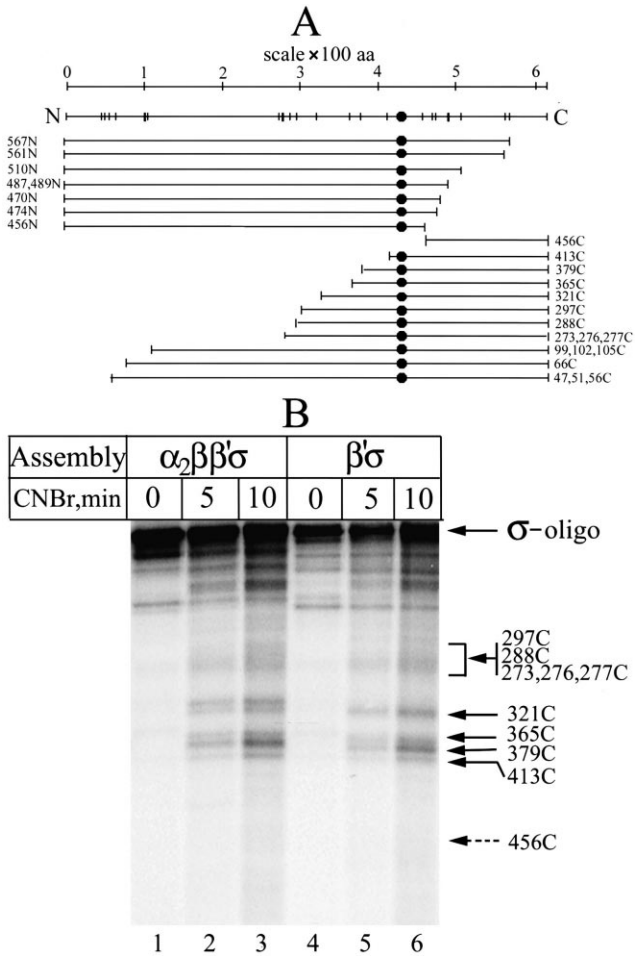

Fig. 2. Mapping of the sites of nt-oligo cross-linking in σ^{70}. A: CNBr degradation map of the 613-amino acid *E. coli* σ^{70} subunit. Vertical bars mark Met positions. The solid circle indicates the presumed position of the labeled oligonucleotide. The cleavage products are annotated by positions of corresponding Met residues and letters indicating N- and C-terminal series. B: Autoradiograph of the gradient 7–14% SDS-PAGE showing products of limited CNBr degradation of σ^{70} cross-linked with nt-oligo in the holoenzyme (lanes 1–3) and σ^{70}β′ complex (lanes 4–6). Arrows mark C-terminal-labeled fragments generated by Met cleavage at the positions shown. The dashed arrow marks the deduced position of the unlabeled 456C fragment. The fragments were identified as described in the text.

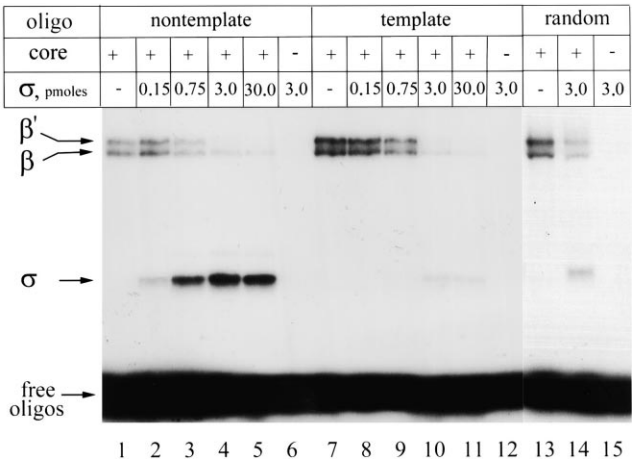

Fig. 3. UV-induced cross-linking of oligos to core and holoenzyme. Samples containing either 1.5 pmol of core polymerase plus indicated quantities of σ^{70} (lanes 1–5, 7–11, 13, 14) or 3 pmol of σ^{70} alone (lanes 6, 12, 15) were incubated for 10 min at 37°C. Then 0.3 pmol of nt- (lanes 1–6), t- (lanes 7–12), or random (lanes 13–15) oligo was added. After 10 min of incubation at 20°C samples were irradiated as described in Section 2. Radiolabeled subunits were separated on 4.5% SDS-PAGE and visualized by autoradiography.

cation of the fragments shown in Fig. 2 was verified by comparison of their pattern with the pattern of fragments labeled with 5- and 7-nucleotide RNAs at the σ^{70} subunit C-terminus (Mustaev, unpublished observation, data not shown). The location of the site of modification can be deduced from the size of the shortest radiolabeled peptide. As can be seen from Fig. 2, the shortest visible peptide modified with the nt-oligo in σ^{70} (either in the holoenzyme or in the $\sigma^{70}\beta'$ complex) is the C-terminal fragment corresponding to cleavage at Met413 (see lanes 2,3 and 5,6). The next Met residue in σ^{70} is Met456. Therefore the site of modification resides between Met413 and Met456, i.e. region 2 of the σ^{70} polypeptide.

3.2. Non-sequence-specific binding of oligonucleotides to core RNAP

Upon examination of the data of Fig. 1, one cannot help noticing that the addition of σ^{70} led to a marked reduction of non-specific cross-linking of oligonucleotides to the large subunits of core enzyme. We explored this phenomenon in more detail in the experiment of Fig. 3 using nt-oligo, t-oligo and a mixture of random oligos as the cross-linking probe. One can see that in the case of core polymerase, there are strong cross-links of all types of oligos tested (lanes 1, 7, 13; see also Fig. 1, lanes 1 and 6) to the β and β′ subunits. The addition of σ^{70} greatly reduced the non-sequence-specific cross-linking of oligos to β and β′.

From these results we conclude that oligos and σ^{70} compete for the same sites on the core polymerase. We located these sites using BrCN cleavage of the modified subunits under single-hit conditions. For the β subunit, a major cross-linking site was mapped between Met1230 and Met1273; for the β′ subunit, the major cross-linking site was between Met29 and Cys58 (data not shown).

4. Discussion

The principal conclusion of this study is that interaction with the isolated β′ subunit is sufficient for inducing conformational change in σ^{70} unmasking its ssDNA binding center. The non-template promoter DNA fragment cross-links to the $\beta'\sigma$ subassembly within region 2 of the σ^{70} polypeptide which was implicated in specific recognition of the non-template promoter DNA strand in numerous studies [10,11,20]. The next step of this study, which is in progress, is to determine which of the four structural modules of β′ induces conformational change in σ^{70} and whether the remaining part of the β′ polypeptide is folded into a distinct structure in the $\beta'\sigma$ subassembly.

We also characterized sites on the core enzyme which bind ssDNA in a non-sequence-specific manner. The core-DNA contact sites were mapped between Met29 and Cys58 of β′ and Met1230 and Met1273 of β. Previously, the β site has been implicated in catalytic functions [19,21,22]. Both sites have been implicated in interactions with DNA template duplex and RNA product [23]. These sites do not cross-link to oligos upon σ^{70} addition. One can speculate that these sites are either sterically blocked by σ^{70} or undergo conformational change induced by σ^{70}. This finding is in accord with the early suggestion that core polymerase sites which are masked by σ^{70} interact with RNA product and/or DNA template after σ is released during transition from initiation to elongation [24].

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