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Region 3.2 of the σ factor controls the stability of rRNA promoter complexes and potentiates their repression by DksA

Danil Pupov[1], Ivan Petushkov[1], Daria Esyunina[1], Katsuhiko S. Murakami[2] and Andrey Kulbachinskiy[1,*]

[1]Institute of Molecular Genetics, Russian Academy of Sciences, Moscow 123182, Russia and [2]Department of Biochemistry and Molecular Biology, The Center for RNA Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA

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ABSTRACT

The σ factor drives promoter recognition by bacterial RNA polymerase (RNAP) and is also essential for later steps of transcription initiation, including RNA priming and promoter escape. Conserved region 3.2 of the primary σ factor ('σ finger') directly contacts the template DNA strand in the open promoter complex and facilitates initiating NTP binding in the active center of RNAP. Ribosomal RNA promoters are responsible for most RNA synthesis during exponential growth but should be silenced during the stationary phase to save cell resources. In *Escherichia coli*, the silencing mainly results from the action of the secondary channel factor DksA, which together with ppGpp binds RNAP and dramatically decreases the stability of intrinsically unstable rRNA promoter complexes. We demonstrate that this switch depends on the σ finger that destabilizes RNAP–promoter interactions. Mutations in the σ finger moderately decrease initiating NTP binding but significantly increase promoter complex stability and reduce DksA affinity to the RNAP–rRNA promoter complex, thus making rRNA transcription less sensitive to DksA/ppGpp both *in vitro* and *in vivo*. Thus, destabilization of rRNA promoter complexes by the σ finger makes them a target for robust regulation by the stringent response factors under stress conditions.

INTRODUCTION

Promoter recognition is a double-sided problem for RNA polymerase (RNAP). First, promoters should be bound sufficiently strongly to allow site-specific DNA melting and transcription initiation. Second, specific RNAP–promoter contacts must be labile enough to be torn apart during transition to productive RNA synthesis. In bacteria, transcription initiation is performed by the holoenzyme of RNAP consisting of the catalytic core enzyme and a specificity subunit, the σ factor. The primary σ factor responsible for transcription of housekeeping genes binds two main promoter elements, –10 and –35, through its conserved regions 2 and 4, and additional extended -10 and discriminator elements through regions 3.0 and 1.2 (1,2). The persisting contacts of σ with these elements result in the extension of the melted promoter region during initial RNA synthesis, accompanied by DNA scrunching and accommodation of excess DNA within the DNA binding cleft of RNAP (3–5). DNA scrunching was proposed to serve as one of driving forces for promoter escape through formation of a stressed complex, which can be relaxed by either RNAP backtracking and release of abortive RNA transcripts, or breakage of RNAP–promoter contacts and transition to RNA elongation (reviewed in (6)).

In the open promoter complex, region 3.2 of the primary σ factor (σ3.2, 'σ finger') directly contacts the template promoter strand upstream of the transcription start site (Figure 1) (7,8). These contacts were proposed to position the DNA template properly in the active site and facilitate initiating NTP (*i*NTP) binding (7,9,10). It was therefore natural to suppose that these σ-DNA contacts may stabilize RNAP–promoter complexes. The same region also blocks RNA extension beyond 4–6 nucleotides and should be removed from its position during transition from the open promoter complex to productive RNA elongation (Figure 1) (8,9,11,12). These σ-RNA contacts were proposed to trigger σ dissociation and stimulate promoter escape by RNAP (9,10,12,13).

Ribosomal RNA promoters account for most transcription in exponentially dividing cells but reveal several unusual features when compared with other promoters. These include the presence of an upstream (UP) promoter element

*To whom correspondence should be addressed. Tel: +7 499 196 0015; Fax: +7 499 196 0015; Email: akulb@img.ras.ru

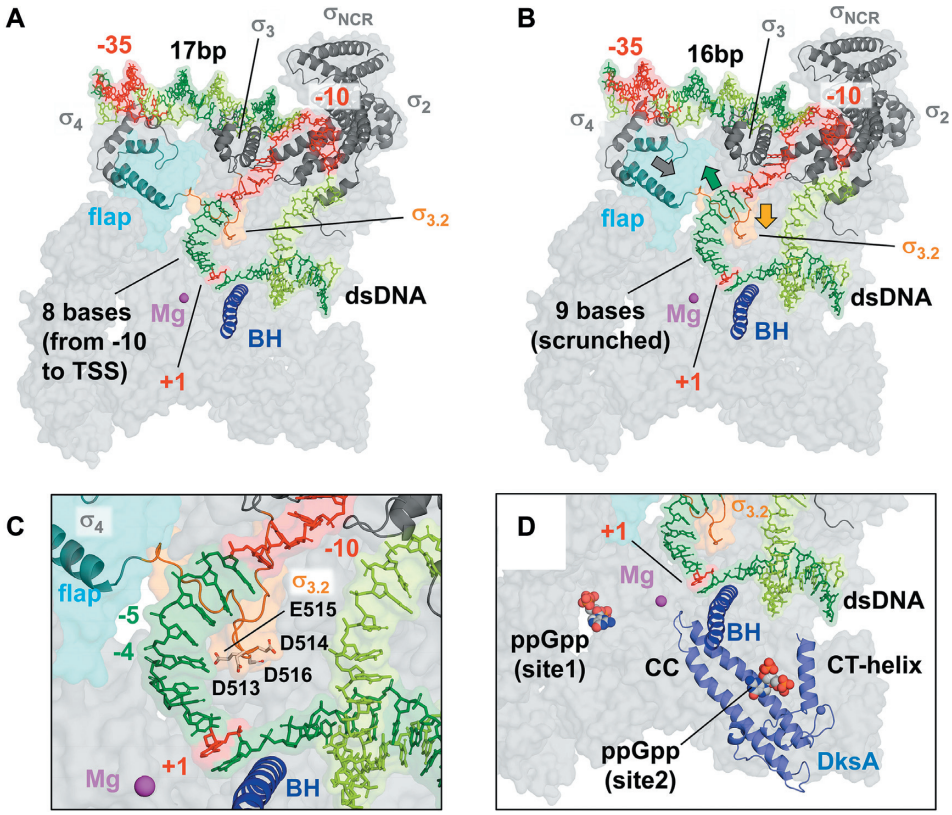


Figure 1. Structural models of open promoter complexes. The models of complexes containing T7A1 (**A**) and *rrnB* P1 (**B**) promoters with scrunched DNA were constructed by using crystal structures of *E. coli* transcription initiation complexes containing short RNAs (panel A, 4 nt RNA, PDB: 4YLN; panel B, 5 nt RNA, PDB: 4YLP) (8), which were removed to imitate corresponding open promoter complexes. RNAP holoenzyme is depicted as partially transparent gray surface. For clarity, the β subunit is removed except for the flap domain. Template and nontemplate DNA strands are depicted in dark and light green stick representation, respectively, with the –35/–10 elements and the transcription start site (+1) shown in red. In panel B, the directions of possible movements of σ domain 4 and the σ finger required for binding the *rrnB* P1 promoter containing 16 bp spacer are indicated by gray and yellow arrows; scrunching of the template DNA strand due to the longer distance from the -10 element to the transcription start site (TSS) is indicated by a green arrow. (**C**) A magnified view of the σ finger in the *rrnB* P1 promoter complex (from panel B). Acidic residues of the σ finger are depicted as sticks and labeled. These residues locate template DNA bases at the –4 and –5 positions. (**D**) A model of the *rrnB* P1 promoter complex with DksA and ppGpp was constructed by superimposing the *rrnB* P1 promoter complex (B) with the crystal structure of the RNAP-DksA/ppGpp complex (PDB: 5VSW) (17). DksA (lilac ribbon) and ppGpp (CPK representation) are labeled. Domains and motifs of RNAP and transcription factors playing key roles in the open complex formation are depicted as ribbons and labeled (BH, bridge helix; σ_{NCR}, nonconserved region of σ^{70} between regions 1.2 and 2.1; CC, N-terminal coiled-coil domain of DksA; CT-helix – C-terminal helix of DksA).

contacted by the α subunits of the core RNAP, a suboptimal 16 bp spacer between the –35 and –10 elements, a suboptimal discriminator sequence, and the transcription start site located 9 bp downstream of the –10 hexamer, instead of 7 bp in most promoters (14,15). As a result rRNA promoters, including most widely studied *rrnB* P1 in *Escherichia coli*, form very dynamic complexes with the RNAP holoenzyme, which is essential for rapid transcription initiation and for drastic changes in rRNA production depending on growth conditions (15–20).

In the exponential phase, the rRNA promoters are highly active due to the presence of nearly consensus promoter elements, high levels of negative DNA supercoiling and high intracellular NTP concentrations. Under these conditions, the unusual architecture of promoter complexes likely increases their activity by facilitating promoter escape by RNAP (6,20). Intriguingly, rRNA promoter complexes were shown to adopt a scrunched conformation even in the absence of NTPs, likely as a result of weakened –10 region contacts and the suboptimal –10/–35 spacer length (20). While the scrunched state was not directly linked to the instability of promoter complexes, it was proposed to stimulate promoter escape, by decreasing the extent of abortive synthesis and, possibly, facilitating displacement of the σ finger from the RNA exit channel (20).

During the stationary phase, the rRNA promoters are downregulated due to drop in DNA supercoiling and NTP concentrations, and the action of the stringent response factors, DksA and ppGpp (21–24). DksA is found in many proteobacteria including *E. coli* and clinically important pathogens (25,26); together with ppGpp it decreases the stability of rRNA promoter complexes and thus effectively prevents rRNA transcription (15,17–19,27–30). Structural and biochemical studies suggested that DksA allosterically affects RNAP-DNA interactions by binding within the secondary channel of RNAP, and ppGpp potentiates DksA action by optimizing DksA position within RNAP and increasing its affinity to RNAP (17,18,29,30).

In this study, we demonstrate that the σ finger negatively affects interactions of the *E. coli* σ^{70} RNAP holoen-

zyme with the *rrnB* P1 promoter, by decreasing promoter complex stability and making it a target for repression by DksA/ppGpp. Mutations in the σ finger greatly stabilize the *rrnB* P1 promoter complex *in vitro*, with only moderate effects on *i*NTP binding and transcription start site selection, and activate rRNA transcription *in vivo*. Together, our data reveal an unexpected role of the σ finger in promoter complex formation and suggest that this region is involved in the control of the activity of unstable promoters under changing growth conditions.

MATERIALS AND METHODS

Proteins and DNA templates

Escherichia coli RNAP core enzyme, wild-type and mutant σ^{70} factors were obtained as previously described (10). The plasmids containing wild-type or mutant *rrnB* P1 variants were obtained by cloning corresponding PCR products into the pTZ19 vector containing a *hisT* terminator. The transcription templates were purified using standard protocol for maxi-prep purification. For the primer extension experiments, the samples were additionally treated with RNaseA and proteinase K to remove all rRNA contaminations. Linear DNA fragments containing the *rrnB* P1 or T7A1cons promoters were obtained by PCR. The promoter sequences are shown in Supplementary Figure S1.

In vitro transcription

Promoter complexes were prepared by incubating core RNAP (100 nM final concentration) with wild-type or mutant σ^{70} factors (500 nM) in transcription buffer (40 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$ and 150 mM KCl, unless otherwise indicated) for 5 min at 37°C, followed by the addition of supercoiled plasmid DNA (15 nM) or linear DNA fragments (15 nM). The samples were incubated for 5 min at 37°C. DksA (3 μM) and ppGpp (200 μM) were added when indicated, and the samples were incubated for additional 5 min at 37°C. NTP substrates were added at the following final concentrations: 200 μM ATP, CTP, GTP, 5 μM UTP, 25 μM CpA, with addition of α-[^{32}P]-UTP. For mapping of the transcription start site (Figure 3), CpA was omitted from the reactions. For measurements of promoter complex stability on the *rrnB* P1 templates, heparin was added to 10 μg/ml for indicated time intervals before NTP addition, and rifapentin was added together with NTPs to 5 μg/ml. The reactions were stopped after 5 minutes at 37°C by the addition of an equal volume of stop-buffer containing 8 M urea and 20 mM EDTA. RNA products were separated by 15% denaturing PAGE and analyzed by phosphorimaging (Typhoon 9500, GE Healthcare). To calculate the observed rate constants (k_{obs}) for promoter complex dissociation the data were fitted to the one-exponential equation:

$A = A_0 \times \exp(-\text{time} \times k_{obs})$, where A is RNAP activity (the amount of RNA synthesized at a given time point after heparin addition) and A_0 is activity measured in the absence of heparin. The promoter complex half-life times were calculated using the formula $t_{1/2} = \ln 2/k_{obs}$.

For T7A1cons, promoter complex stabilities were measured using a molecular beacon assay as described in (31,32). Promoter complexes were formed with tetramethylrhodamine-labeled derivatives of the wild-type or 513–516A σ^{70} subunits (containing a unique cysteine residue introduced at σ position 211) in transcription buffer containing 40 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$, 40 mM KCl and 0.01% Tween-20, and heparin was added to 100 μg/ml. After the addition of heparin, fluorescence intensity was measured every second, the relative fraction of the open complex was calculated by dividing the fluorescence intensity at each time point by the starting intensity before competitor addition, and the data were fitted to the one-exponential equation (31,32).

Apparent K_Ms for *i*NTPs for the *rrnB* P1 promoter were measured on the supercoiled plasmid template in reactions containing ATP and CTP. One of the two NTPs was taken at 1 mM concentration and the concentration of the other was varied from 10 μM to 3 mM; γ-[^{32}P]-ATP was added to label the dinucleotide RNA product. The reactions were terminated after 1 minute at 37°C, and the samples were analyzed by 30% PAGE. The data were fitted to the Michaelis–Menten equation:

$A = A_{max} \times [NTP]/(K_M + [NTP])$, where A is the amount of synthesized RNA and A_{max} is the amount of RNA at saturating NTP concentrations.

For measurements of apparent DksA affinities, DksA was added at different concentrations (from 100 nM to 10 μM) to preformed promoter complexes with linear DNA templates, either in the absence or in the presence of ppGpp (200 μM), in transcription buffer containing 40 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$ and 40 mM KCl with 100 μg/ml BSA. NTP substrates were added together with rifapentin (5 μg/ml) and transcription was performed for 5 min at 30°C. RNA products were analyzed as described above and the data were fitted to the hyperbolic equation:

$A = A_{max} \times (1 - [DksA]/(K_{d,app} + [DksA]))$, where A is RNAP activity at a given DksA concentration and A_{max} is RNAP activity in the absence of DksA.

Primer extension

The products of *in vitro* transcription were treated with 25 units of DNaseI (Thermo Scientific) for 15 min at 37°C and deproteinized with phenol and chloroform. RNA was ethanol precipitated and dissolved in 1^x RT reaction buffer (Thermo Scientific). 1 pmol of 5′-[^{32}P]-labeled primer, corresponding to positions +12/+35 of the RNA transcript relative to the main transcription initiation site in the *rrnB* P1 promoter, was added; the samples were incubated for 7 min at 80°C and then slowly cooled down to 30°C. One hundred units of RevertAid Reverse Transcriptase and 400 mM dNTPs were added to the mixture and the samples were incubated for 20 min at 50°C. The reaction was stopped by the addition of an equal volume of formamide and heated for 3 min at 95°C. The primer extension products were analyzed by 17% PAGE.

In vivo assays

MG1655 or MG1655 *dksA*$^-$ *E. coli* cells were co-transformed with plasmids pET28_rrnBP1_luxCDABE and pBAD_rpoD encoding the bacterial luciferase operon from *Photorhabdus luminescence* under the control of the *rrnB*

P1 promoter and the σ^{70} factor (wild type or mutant) under the control of the arabinose promoter, respectively, in the presence of kanamycin (50 μg/ml) and ampicillin (100 μg/ml). The MG1655 *dksA*$^-$ strain was kindly provided by S. Proshkin and A. Mironov (33). The cells were grown in 1 ml volume of the LB broth in 24-well plates at 32°C at 400 rpm for 24 h; 10 μl of each culture were then inoculated into 1 ml of fresh broth, the expression of σ^{70} was induced by the addition of arabinose to 0.02% or 0.2% final concentration and cell growth was continued under the same conditions. Luminescence measurements were performed after 24 h in 100 μl samples in black plates in the Modulus luminometer (Turner BioSystems, USA). Optical density (OD_{600}) measurements were performed in 3–10-fold diluted samples in 24-well plates in the Tecan plate reader. Normalized luminescence values were obtained by dividing the raw luminescence data by the OD_{600} value in each sample. 100 μl of each sample were also taken for western blot analysis (Supplementary Figure S4). The growth curves (Supplementary Figure S5) were measured in the Tecan plate reader at 32°C with constant shaking (200 rpm) and OD_{600} detection every 10 min.

RESULTS

Stabilization of the *rrnB* P1 promoter complex by σ finger mutations

Previously, we demonstrated that the σ finger is involved in several steps of transcription initiation, including *i*NTP binding, abortive RNA synthesis and promoter escape by RNAP (10,12,13). However, those studies were performed with stable promoters and therefore did not assess a possible role of the σ finger in the formation and stabilization of promoter complexes. In this study, we chose the unstable *rrnB* P1 promoter to test the prediction that contacts of the σ finger with the template strand in the single-stranded DNA region may be important for promoter complex stability. We analyzed three σ^{70} variants with mutations in region 3.2: a deletion of seven amino acid residues (Δ513–519), a quadruple substitution of four acidic residues (D513, D514, E515, D516) in this region with alanines (513–516A), and a double substitution of two of these residues (514,516A).

The promoter activity was analyzed on supercoiled plasmid templates containing the *rrnB* P1 promoter placed 88 nt upstream of a *hisT* terminator. Another transcript monitored in our assays was RNA I (108–110 nt) encoded by the *ori* region of the plasmid; this promoter forms stable complexes with the RNAP holoenzyme and can serve as an internal control for open complex formation (34). To measure promoter complex lifetimes, preformed promoter complexes of wild-type or mutant RNAP holoenzymes were incubated with a DNA competitor heparin, followed by the addition of NTPs and rifapentin. The addition of rifapentin limited transcription to a single round of RNA synthesis in all samples, including control reactions without heparin addition. Previously, it was shown that mutations in the σ finger impair transcription initiation at low NTP concentrations at various promoters including *rrnB* P1; these defects could be partially suppressed in the presence of a two-nucleotide primer corresponding to the starting point of transcription in *rrnB* P1 (CpA, promoter positions –1/+1) (10). Thus, to minimize possible effects of the mutations on *i*NTP binding, all *in vitro* experiments were performed at sufficiently high NTP concentrations (200 μM ATP, CTP, GTP, 5 μM UTP), with the addition of CpA primer.

The RNA I promoter complexes remained stable during the course of the experiment for all RNAP variants ($t_{1/2}$ > 900 s, Figure 2A). In agreement with published data, the wild-type RNAP holoenzyme formed unstable complexes with *rrnB* P1, with half-life time of 55 s (Figure 2A). Surprisingly, deletion of the σ finger (Δ513–519) significantly increased promoter complex stability (~5-fold, $t_{1/2}$ = 285 s), and the four-alanine substitution (513–516A) had even a greater effect on the *rrnB* P1 complexes (~15-fold increase in the stability, $t_{1/2}$ = 830 s) (Figure 2A and B). The double-alanine substitution had an intermediate effect on the promoter complex half-life (2.3-fold increase in the stability). This suggested that, contrary to the expectations, contacts of the σ finger with DNA destabilize the *rrnB* P1 promoter complexes.

Interestingly, previous studies demonstrated that mutations in the σ finger decrease RNAP activity on various promoters including *rrnB* P1 (10), while in the experiments presented above the activities of mutant RNAPs were comparable or even higher than the activity of the wild-type RNAP holoenzyme (Figure 2). We supposed that this could be due to the presence in the reaction of heparin and rifapentin that limited transcription initiation by the wild-type holoenzyme, especially from the unstable *rrnB* P1 promoter; when added together with NTPs, both compounds would block transcription initiation by RNAP molecules not engaged into catalytically competent open complexes. Indeed, when transcription initiation was performed in the absence of heparin, the activity of mutant RNAP holoenzymes was lower than the activity of the wild-type holoenzyme, while the situation was reversed in the presence of heparin, due to the strong inhibition of wild-type RNAP (Supplementary Figure S3, (10), see also Figure 4 below).

Effects of the σ finger mutations on transcription start site selection

Previously, certain substitutions in *rrnB* P1 were shown to shift the transcription start site to a position 6 bp downstream of the –10 hexamer, similarly to other promoters (in contrast to 9 bp observed in wild-type *rrnB* P1). Most such substitutions either increased the length of the –35/–10 promoter spacer or stabilized interactions of σ with the discriminator region (16,20). Since mutations in the σ finger likely change the σ-DNA contacts around the transcription start site (see Discussion), we tested whether these mutations might also affect start site selection in *rrnB* P1. The 5′-ends of RNA transcripts were mapped by the extension of a primer located downstream of the transcription initiation site with reverse transcriptase. As expected, the major start site detected for the wild-type RNAP holoenzyme was located 9 bp of the –10 element (Figure 3). The σ3.2 deletion resulted in a partial shift of the start site to the adenine residue located 6 bp downstream of the –10 element. At the same time, the 513–516A substitution had essentially no effect on the start site selection (Figure 3). Therefore, stabi-

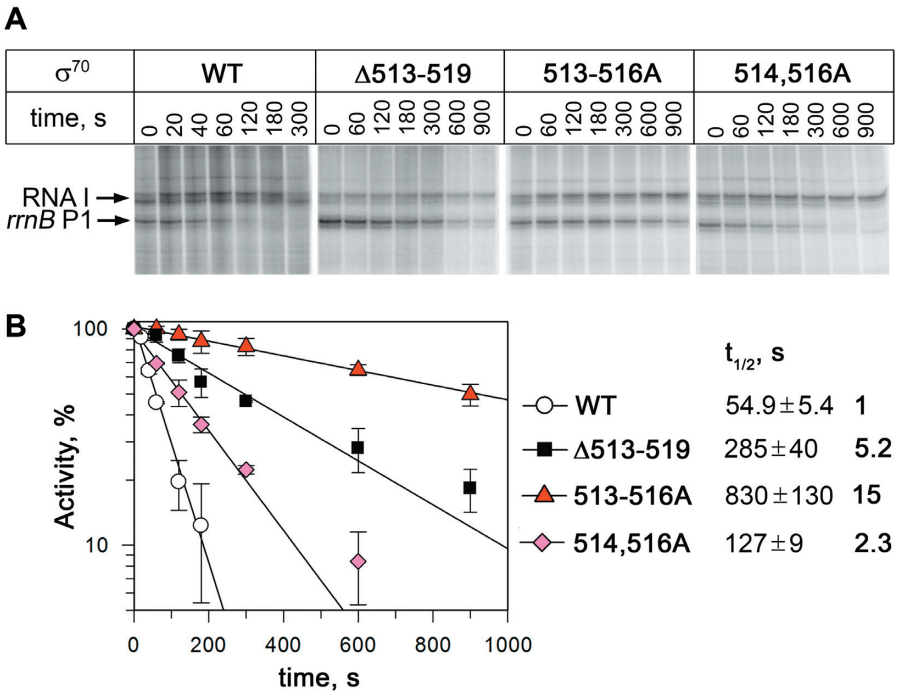


Figure 2. Stabilities of promoter complexes formed by the wild-type and mutant RNAPs. (**A**) Sensitivity of *rrnB* P1 promoter complexes to heparin. Preformed promoter complexes were incubated with heparin for indicated time intervals, followed by the addition of NTPs and rifapentin. (**B**) Kinetics of promoter complex dissociation for wild-type and mutant RNAPs. The half-life times of promoter complexes for each RNAP are shown on the right (mean values and standard deviations from three independent experiments).

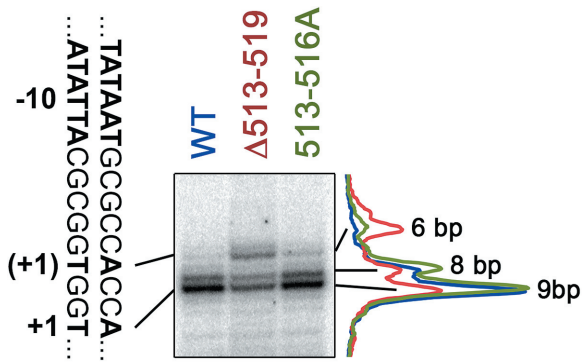


Figure 3. Effects of the σ finger mutations on start site selection. Transcription start points were determined in primer extension reactions as described in Materials and Methods. Positions of the transcription start sites 9, 8 or 6 bp downstream of the –10 hexamer observed for the wild-type and mutant RNAPs are indicated. The scanned profiles of the primer extension products for each RNAP are shown on the right.

lization of the *rrnB* P1 promoter complex by the σ3.2 mutations does not necessarily require shifting of its transcription start site.

Role of the σ finger in *i*NTP binding

Transcription initiation from rRNA promoters was previously shown to be highly sensitive to intracellular NTP concentrations, which greatly vary at different growth conditions (23). *In vitro* experiments also demonstrated that full-length RNA synthesis initiated from rRNA promoters is sensitive to the concentration of +1 and +2 *i*NTPs (ATP and CTP in *rrnB* P1) (21–23). Since mutations in the σ finger were previously shown to increase apparent Michaelis constants ($K_{M,app}$) for *i*NTPs on other promoters (10,13), we measured $K_{M,app}$ values for initiating substrates for the wild-type and mutant RNAP holoenzymes on *rrnB* P1.

Table 1. Apparent K_M values for *i*NTPs on the *rrnB* P1 promoter for the wild-type and mutant RNAPs

	ATP+CTP → pppApC	
RNAP	K_M, ATP (μM)	K_M, CTP (μM)
WT	78 ± 27 **1**	53 ± 18 **1**
Δ513–519	227 ± 22 **2.9**	248 ± 50 **4.6**
513–516A	236 ± 16 **3.0**	106 ± 21 **2.0**
514,516A	154 ± 10 **2.0**	62 ± 14 **1.2**

We first determined $K_{M,app}$ for the +1 (ATP) and +2 (CTP) nucleotides in the reaction of dinucleotide synthesis, when the concentration of one nucleotide was varied and the second was taken at an excess. It was found that the $K_{M,app}$ values were not very much different from those previously measured for stable promoters, with a somewhat higher value for the second *i*NTP: 78 μM for +1 ATP and 53 μM for +2 CTP in the case of *rrnB* P1 (Table 1); 190 μM for +1 ATP and 10 μM for +2 UTP in the case of T7A1 (35).

We then determined apparent K_Ms for ATP and CTP in the reaction of full-length RNA synthesis, when the con-

centration of one nucleotide was varied and the remaining three NTPs were kept constant. Under these reaction conditions, the resulting $K_{M,app}$ values were lower (11 μM for ATP and 1.7 μM for CTP; Supplementary Figure S2) than those measured in the dinucleotide reaction. This could be expected since these values are composite K_Ms for both the initiation and elongation steps of transcription. It should be noted that the apparent K_M for +1 ATP measured in this way is lower than the previously reported values but still comparable with published data (e.g. 20 μM in (22)). Furthermore, these data demonstrate that the composite K_Ms measured in the full-length RNA synthesis do not necessarily correspond to the K_Ms of *i*NTPs and may likely depend on the specific reaction conditions (see Discussion).

We further tested whether mutations in the σ finger had any effects on the *i*NTP K_Ms. All three mutants (the Δ513–519 deletion and alanine substitutions) moderately affected $K_{M,app}$ for iNTPs in the dinucleotide reaction (2–3-fold increase at maximum) (Table 1). The σ finger deletion also increased *i*NTP K_Ms in the full-length RNA synthesis while the four-alanine substitution had no significant effect in this reaction (Supplementary Figure S2). In agreement with this, full-length RNA synthesis by the mutant RNAP holoenzyme from the *rrnB* P1 promoter was compromised at low NTP concentrations, and this defect could be compensated by the addition of the CpA primer (10). The overall conclusion is that the σ finger facilitates *i*NTP binding in the case of rRNA promoters, similarly to previously studied stable promoters (10,13), despite its negative impact on the promoter complex stability.

Regulation by DksA and ppGpp

In vivo, the activity of rRNA promoters is controlled by the stringent response factors DksA and ppGpp that drastically destabilize rRNA promoter complexes and redirect RNAP to other, more stable cellular promoters (see Introduction). We therefore tested whether mutations in the σ finger would modulate the effects of the stringent response factors on the *rrnB* P1 activity. We first analyzed the influence of DksA and ppGpp on RNAP activity measured in the absence of competitors. It was found that in the plasmid-directed transcription assay, the synthesis of RNA I by the wild-type RNAP holoenzyme remained unaffected by either DksA or ppGpp, or was even slightly stimulated when both factors were added simultaneously (Figure 4A, lanes 1–4, Figure 4B). Similarly, RNA I synthesis by the 513–516A (Figure 4A, lanes 5–8) or Δ513–519 (lanes 9–12) mutant RNAP holoenzymes was only slightly affected by DksA or ppGpp. In contrast, each of the factors decreased the activity of wild-type RNAP holoenzyme on *rrnB* P1 about 2-fold, and the addition of both of them led to ∼6-fold transcription inhibition (Figure 4C; for each RNAP, the *rrnB* P1 activity was normalized to the RNA I synthesis). RNAP with the σ finger deletion (Δ513–519) was only slightly more resistant to DksA/ppGpp under these conditions. At the same time, the four-alanine mutant (513–516A) was significantly more active in the presence of either DksA, or ppGpp, or both factors ($P < 0.01$; Figure 4C).

We then analyzed the decay of the *rrnB* P1 activity in the presence of heparin. Preformed promoter complexes were supplemented with DksA and ppGpp, followed by the addition of heparin, and the residual RNAP activity was measured over time. Under these conditions the wild-type RNAP holoenzyme revealed no *rrnB* P1 activity at all time points, with unchanged levels of the RNA I synthesis, indicating that the lifetime of *rrnB* P1 promoter complexes was < 20 s (Figure 5). At the same time, mutant RNAPs with the σ finger deletion or alanine substitutions in this region retained a substantial level of the *rrnB* P1 activity up to 60 s incubation time with heparin. Thus, the stabilization effect of the σ finger mutations on the *rrnB* P1 promoter complexes is also observed in the presence of DksA and ppGpp. Similarly to the experiments performed in the absence of DksA and ppGpp (see above, Figure 2 and Supplementary Figure S3), the mutants were much more active than the wild-type RNAP holoenzyme in the presence of heparin, even though their absolute activities in the absence of DNA competitors were comparable to or lower than the activity of wild-type RNAP holoenzyme (see Figure 4).

To get a more detailed view on the role of the σ finger in promoter complex stability and RNAP holoenzyme sensitivity to DksA, we compared promoter complex half-lives for wild-type and 513–516A RNAP holoenzymes on three promoters: wild-type *rrnB* P1, C-2T *rrnB* P1 and T7A1cons (see Supplementary Figure S1 for promoter sequences), both in the absence and in the presence of DksA. The C-2T *rrnB* P1 promoter is a previously described *rrnB* P1 variant that forms more stable complexes with the wild-type RNAP holoenzyme (16). T7A1cons is a consensus variant of the T7A1 promoter that is characterized by a low efficiency of promoter escape, in particular, in the case of RNAPs with mutations in σ region 3.2 (10,13). Since this promoter forms stable complexes with RNAP holoenzyme, we were able to measure their stability directly, by using the previously described molecular beacon assay (31,32). For this purpose, RNAP holoenzymes containing fluorescently-labeled σ^{70} factor variants (either wild-type or 513–516A) were incubated with linear T7A1cons promoter templates, leading to a significant increase in the fluorescence intensity. Promoter complex dissociation was then monitored by the decrease in fluorescence following heparin addition.

The 513–516A substitution increased promoter complex half-lives not only for wild-type *rrnB* P1 but also for the C-2T *rrnB* P1 (∼2.5-fold) and T7A1cons (>10-fold) promoters (Table 2), suggesting that in wild-type σ^{70} region 3.2 may have a general destabilizing effect on promoter complex formation. For all three promoters DksA decreased the promoter complex half-lives, although the effect was smaller in the case of the more stable T7A1cons promoter (<2-fold reduction in the $t_{1/2}$; Table 2). At the same time, the 513–516A substitution greatly increased promoter complex half-lives in the presence of DksA for all three promoters (20-fold or more; Table 2). Therefore, mutations in the σ finger allow RNAP to overcome the negative effect of DksA on promoter complex formation.

Effects of mutations in the σ finger on DksA binding

It was previously demonstrated that interactions of RNAP with the σ factor and with promoter DNA may affect DksA binding (17,36). We therefore tested whether mutations in

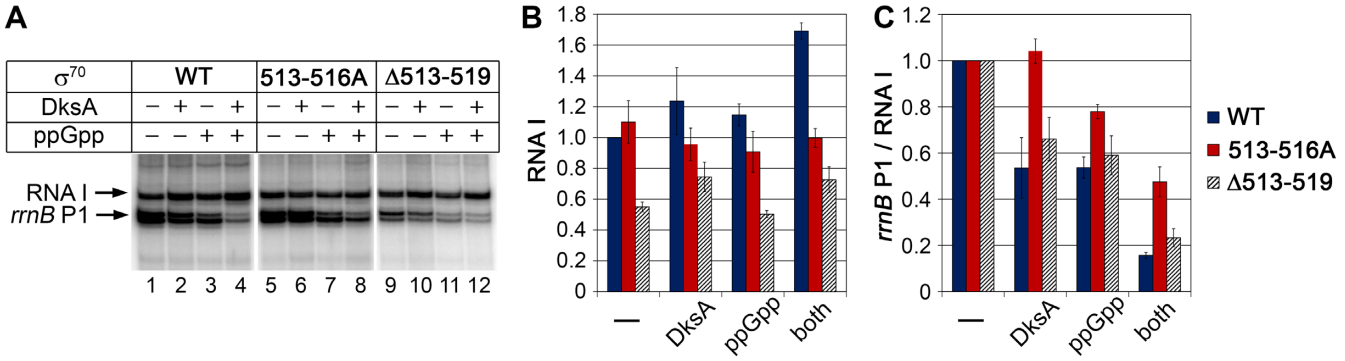


Figure 4. Inhibition of the *rrnB* P1 activity by DksA and ppGpp. (**A**) RNAP activity was measured on the supercoiled *rrnB* P1 template either in the absence of factors or in the presence of DksA and/or ppGpp. (**B**) Effects of DksA and ppGpp on RNA I synthesis. The levels of RNA I transcription are shown relative to the activity of wild-type RNAP holoenzyme measured in the absence of factors. (**C**) Effects of DksA and ppGpp on the *rrnB* P1 activity. For each RNAP, the levels of *rrnB* P1 transcription were normalized to the RNA I transcription and then normalized to this value measured in the absence of DksA/ppGpp. Means and standard deviations from three to four independent experiments are shown.

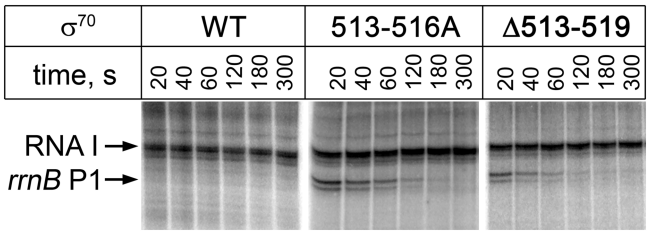
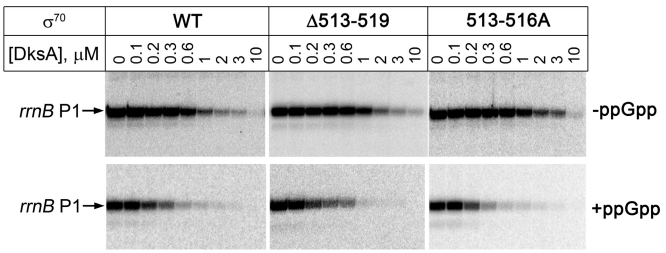


Figure 5. Stabilities of the *rrnB* P1 promoter complexes formed by wild-type and mutant RNAPs in the presence of DksA and ppGpp. Preformed promoter complexes were incubated with DksA and ppGpp, followed by the addition of heparin. NTPs and rifapentin were added after indicated time intervals and transcription was performed for 5 min at 37°C.

Table 2. Effects of DksA on the stability of promoter complexes formed by the wild-type and mutant RNAPs

	$t_{1/2}$ (s)	
Promoter/RNAP	−DksA	+DksA
***rrnB* P1 WT**		
WT	54.9 ± 5.4 **1**	20 ± 2* **1**
513–516A	830 ± 129 **15.1**	228 ± 16* **21**
***rrnB* P1 C-2T**		
WT	200 ± 16 **1**	17 ± 5 **1**
513–516A	514 ± 27 **2.6**	298 ± 45 **17.5**
T7A1cons		
WT	164 ± 14* **1**	92 ± 12* **1**
513–516A	>30 min* **>10**	>30 min* **>20**

The dissociation kinetics were determined from measurements of RNAP activities (*rrnB* P1) or changes in the fluorescence intensity (T7A1cons, molecular beacon assay) at different time intervals after heparin addition. The measurements were performed in transcription buffers containing 150 mM KCl or 40 mM KCl (indicated with asterisks) with supercoiled (for both *rrnB* P1 variants) or linear (for T7A1cons) templates. The numbers in bold indicate fold-changes in the promoter complex half-lives for the 513–516A RNAP relative to the wild-type control. For the mutant RNAP at the T7A1cons promoter, the complexes were stable during the time course of experiment ($t_{1/2}$ > 30 min), so a minimum estimate of changes is indicated.



RNAP	$K_{d,app}$ (nM)	
	-ppGpp	+ppGpp
WT	300 ± 44 **1**	150 ± 34 **1**
Δ513-519	660 ± 125 **2.2**	195 ± 31 **1.3**
513-516A	925 ± 103 **3.1**	150 ± 14 **1.0**

Figure 6. Apparent DksA affinities to wild-type and mutant RNAPs. Transcription was performed on a linear DNA fragment containing the *rrnB* P1 promoter. Preformed promoter complexes were incubated with increasing amounts of DksA either in the absence or in the presence of ppGpp for 5 min at 30°C, followed by the addition of NTPs and rifapentin. Apparent K_d values were calculated from the inhibition curves as described in Materials and methods. Mean values and standard deviations from three independent experiments are shown.

σ region 3.2 may change DksA affinity to promoter complexes, by measuring RNAP activities at increasing DksA concentrations. To enhance the inhibitory effect of DksA on transcription, the reactions were performed with linear DNA templates, at suboptimal temperature (30°C), and in the presence of rifapentin (to block transcription initiation by free RNAP molecules or by closed promoter complexes). It was found that under these conditions DksA completely inhibited the activities of wild-type and mutant RNAP holoenzymes, but with different apparent K_d values (Figure 6). The half-inhibitory concentration of DksA was increased from 300 nM for the wild-type RNAP holoenzyme to 660 and 925 nM for the Δ513–519 and 513–516A RNAPs, ∼2- and 3-fold increase, respectively. Thus, the σ finger mutations not only stabilize *rrnB* P1 promoter complexes but also decrease apparent DksA affinity, which may

contribute to their activation effect on the *rrnB* P1 transcription.

The addition of ppGpp increased apparent DksA affinity to promoter complexes ~2-fold, in agreement with previous reports (17,18). Interestingly, the σ finger mutants revealed the same affinity to DksA in the presence of ppGpp as the wild-type RNAP holoenzyme (Figure 6). Therefore, the defects of these mutants in DksA binding seem to be compensated by the ppGpp addition.

Mutations in the σ finger activate the *rrnB* P1 promoter *in vivo*

The *in vitro* effects of the σ finger mutations on *rrnB* P1-dependent transcription prompted us to investigate their possible action on the *in vivo* activity of rRNA promoters. A reporter luciferase operon (*luxCDABE* from *P. luminescens*) was cloned under the control of the *rrnB* P1 promoter and introduced into *E. coli* strains (either wild type or *dksA*$^-$) containing pBAD plasmids encoding wild-type or mutant σ^{70} variants under the control of an arabinose-inducible promoter. In the absence of arabinose, no or very little expression of plasmid-encoded σ variants was detected by Western blot analysis, in comparison with a control empty plasmid (Supplementary Figure S4, lanes 1–4). In the presence of arabinose (either 0.02% or 0.2%, lanes 5–8 and 9–12), robust expression of plasmid-encoded σ^{70} was detected in the both wild-type and *dksA*$^-$ strains, which was several-fold higher than the expression of endogenous σ^{70}. Comparable levels of expression were observed for the wild-type and mutant σ variants. In subsequent experiments, we used the lower concentration of arabinose (0.02%) to minimize its effects on cell growth.

Analysis of the cell growth kinetics revealed no differences between uninduced strains (no arabinose added) containing pBAD plasmids with either wild-type or mutant σ variants (Supplementary Figure S5, upper panel). Slower growth was observed for *dksA*$^-$ cells but it was also similar for all plasmid variants. When the expression of σ was induced by arabinose, most strains again had similar growth kinetics, with the exception of *dksA*$^-$ cells containing the wild-type pBAD-σ^{70} plasmid that revealed a biphasic growth curve (Supplementary Figure S5, lower panel). Thus, luminescence measurements were performed after 24 h of growth with arabinose, when most cells reached the stationary phase with similar OD_{600} levels (although the *dksA*$^-$/pBAD-σ^{70} cells still had lower OD_{600}). For each strain, the luminescence levels were normalized by corresponding OD_{600} values.

We observed that in the wild-type strain the *rrnB* P1 promoter was significantly activated (up to 5-fold) when either Δ513–519 or 513–516A σ^{70} variants were expressed in the cells, in comparison with wild-type σ^{70} or the empty pBAD vector (Figure 7A). No stimulation was observed in control experiments without arabinose addition confirming that the observed increase in the *rrnB* P1 activity resulted from the expression of mutant σ factors.

We further tested the effects of mutant σ factors on the *rrnB* P1 activity in the *dksA*$^-$ strain. As expected, the activity of *rrnB* P1 at the stationary phase was significantly increased in comparison with the wild-type strain, even without the expression of plasmid-encoded σ^{70} variants (Figure 7B, the '-Ara' data points). In contrast to the wild-type cells, expression of mutant σ^{70} variants did not stimulate but even inhibited (~2-fold) luciferase activity (Figure 7B, the '+Ara' data points). Therefore, stimulation of the *rrnB* P1 activity by the mutant σ factors observed in wild-type cells depended on the presence of DksA. At the same time, the *rrnB* P1 activity was still suppressed by DksA to some extent even in the presence of the mutant σ factors, as evident from comparison of the luminescence levels for the wild-type and *dksA*$^-$ cells expressing mutant σs (compare Figure 7A and B). This could be explained by the retained sensitivity of promoter complexes formed by the mutant RNAP holoenzymes to DksA (see Figures 2, 5 and Table 2) and/or by the activity of endogenous wild-type σ^{70} holoenzyme highly sensitive to DksA.

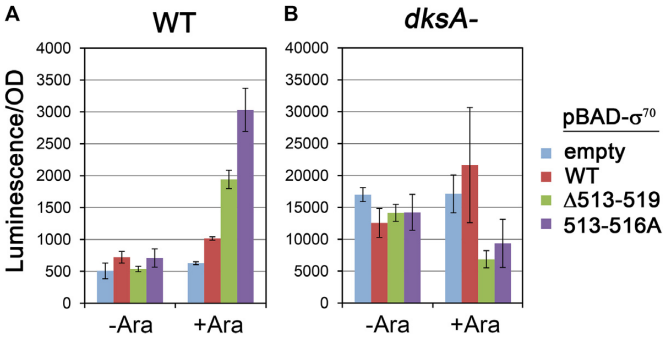


Figure 7. Effects of mutations in the σ finger on the *rrnB* P1 activity *in vivo*. The activity of the reporter *luxCDABE* operon placed under the control of the *rrnB* P1 promoter was compared for wild-type (**A**) and *dksA*$^-$ (**B**) *E. coli* strains in stationary phase as described in Materials and methods. The expression of wild-type (WT) or mutant σ^{70} variants was induced from pBAD-based plasmids by the addition of arabinose (0.02%, +Ara) and compared with uninduced cells (-Ara). Control strain contained an empty plasmid without the *rpoD* gene. The luminescence intensity values were normalized by the optical densities for each strain and are shown in arbitary units. Means and standard deviations from 3 independent experiments are shown.

DISCUSSION

The σ factor of bacterial RNAP holoenzyme is a multifunctional protein engaged in all steps of transcription initiation, starting from promoter recognition and DNA melting to *i*NTP binding, RNA priming and promoter escape (2,37). Remarkably, many of these functions depend on the σ finger formed by σ region 3.2, which first positions the template DNA strand in the open promoter complex and then facilitates σ dissociation after RNA priming has occurred (10,12,13). We now demonstrated that in the case of rRNA promoter complexes, which are renowned by the high rate of transcription initiation and their low stability, the σ finger is in part responsible for this instability and plays as essential role in their regulation by the stringent response factors. We showed that mutations in the σ finger dramatically increase the stability of rRNA promoter complexes both in the absence and in the presence of DksA, and also decrease apparent affinity of DksA to promoter complexes.

From the structural perspective, the σ finger not only positions the template DNA strand at the active site of RNAP, but also makes a barrier for the template entry into the active site during open complex formation (Figure 1) (7,9,17). The DNA binding therefore requires opening of the main channel, including movement of the clamp domain (38). The *rrnB* P1 promoter has a longer DNA segment between the –10 element and the transcription start site so that the DNA binding channel of RNAP should accommodate longer DNA in the open complex compared with other promoters in which this distance is shorter (Figures 1A and B). In addition, the *rrnB* P1 promoter has a short 16 bp spacer between the –10 and –35 elements in comparison with 17–18 bp in most promoters. The shorter spacer brings σ domains 2 and 4 closer to each other in the promoter complex, which may make the σ finger protruding toward the active site (Figure 1B). Both features likely produce a stronger barrier for DNA entry, depending on the σ finger and its interactions with the template DNA strand (17). Indeed, the σ finger was proposed to be partially displaced from its usual position in the open promoter complex in the case of scrunched *rrnB* P1 promoter, due to the 'crowding' with the template DNA strand (Figure 1B) (5,20). Mutations in this region may therefore free more space in the main channel for DNA binding and also alleviate unfavorable interactions between acidic residues in the σ finger and the DNA backbone (Figure 1C), thus explaining their stabilization effects on rRNA promoter complex formation. Importantly, this stabilization is not necessarily accompanied by DNA unscrunching in the open complex and by changes in the transcription start point selection, at least in the case of alanine substitutions in the σ finger (Figure 3). At the same time, the Δ513–519 deletion partially relaxed the scrunched state (the transcription start site was shifted upwards) suggesting that the σ finger may normally promote DNA scrunching during open complex formation.

Mutations in the σ finger make transcription from *rrnB* P1 much less sensitive to inhibition by DksA and ppGpp. The observed stabilization of rRNA promoter complexes by these mutations is likely significant under *in vivo* conditions, by making rRNA transcription more resistant to competing more stable cellular promoters and nonpromoter DNA sites (mimicked by the addition of heparin in our *in vitro* experiments). Indeed, we observed that expression of mutant σ factors stimulated the activity of *rrnB* P1 at the stationary phase *in vivo*, and that this effect was dependent on the presence of DksA in the cell (Figure 7). Remarkably, expression of the mutant σ variants did not stimulate but even inhibited the *rrnB* P1 activity in the *dksA*-strain *in vivo*. Indeed, the σ finger mutations could have been expected to have a general negative effect on RNAP activity because they impair *i*NTP binding and RNA priming (see below).

From the structural point of view, DksA binding, in particular interactions between its C-terminal helix and the β lobe domain of RNAP core enzyme, may prevent clamp opening required for DNA entry. In addition, DksA-induced changes in the bridge-helix conformation may affect positioning of the template strand in the active site thus preventing formation of stable open complexes (Figure 1D) (17). In converse, mutations in the σ finger may facilitate template DNA binding (see above) and thus make transcription less sensitive to DksA. Furthermore, the drop in the apparent affinity of DksA to promoter complexes observed for the mutant RNAPs suggests that the σ finger mutations may indirectly affect DksA binding, by stabilizing promoter complexes. Indeed, we recently demonstrated that DksA affinity to a promoter complex depends on its strength/stability, with stable complexes having weaker DksA affinity in comparison with the unstable *rrnB* P1 (17).

Interestingly, the affinity of DksA to the RNAP holoenzymes containing mutant σ factors was restored in the presence of ppGpp, which was previously shown to form a part of the binding site for DksA in the secondary channel (ppGpp site 2) (Figure 1D) (17,18). In particular, ppGpp binding at site 2 restores the conformation of the β′ rim helix and the β lobe domain in the RNAP-DksA complex and also positions the tip of the coiled-coil domain of DksA closer to the active site (17). In addition, ppGpp binding at site 1 at the interface between β′ and ω subunits (Figure 1D) may promote DksA binding by decreasing promoter complex stability. However, the σ finger mutations can still stabilize the *rrnB* P1 promoter complex and thus activate rRNA transcription in the presence of both DksA and ppGpp.

Similar to other promoters, the σ finger is important for *i*NTP binding in the case of *rrnB* P1 since mutations in this region increased apparent K_Ms for both *i*NTPs and decreased RNAP activity in the absence of DNA competitors (Table 1, Figure 4, Supplementary Figure S2) (10,13). Therefore, mutations in the σ finger may affect template DNA positioning in such a way that the binding of *i*NTPs is impaired. Alternatively, these mutations may allosterically change the active site and/or DNA-binding clamp conformations through other regions of RNAP, including flexible regions switch3 and switch2 which contact, respectively, the σ finger and the template strand immediately downstream of the active site (10,35). It should be noted, however, that the negative effects of the mutations on *i*NTP binding do not prevent them from activating transcription from *rrnB* P1 (in comparison with the wild-type σ^{70}) in the presence of stringent response factors both *in vitro* and *in vivo* (Figures 5 and 7). While dinucleotide primers were used to suppress the *i*NTP binding defects of the mutations *in vitro*, their activity *in vivo* may be supported by higher NTP concentrations, or RNA priming with nanoRNAs produced during transcription initiation from other promoters (39,40). Furthermore, apparent K_Ms for *i*NTPs at *rrnB* P1 measured in the reaction of dinucleotide synthesis demonstrated that these values are comparable with those measured for stable promoters. It is therefore likely that the previously reported strong NTP dependence of transcription initiation from rRNA promoters (23) is not explained by inefficient *i*NTP binding but may result from other promoter complex properties—probably, their low stability—which make *i*NTP binding the rate-limiting step in transcription.

In conclusion, our study adds another level of complexity to the regulation of open complex formation and the role of the σ factor in this process. We show that σ not only provides a binding platform for DNA sequence recognition by RNAP holoenzyme but can also destabilize RNAP-DNA interactions. The rRNA promoter complex stability depends on a complex interplay between the σ finger, specific promoter elements that position DNA in an unusual

distorted conformation, and the action of DksA/ppGpp. Region 3.2 that forms the σ finger is conserved in principal σ factors but has diverse sequences or is even completely absent in alternative σs (e.g. group 4 σ^{24} and σ^{19} in *E. coli*). Given its essential functions and the conserved structure of promoter complexes formed by RNAP holoenzymes containing the σ^{70}-family factors (2,41), it is likely that unrelated protein sequences can take its role in alternative σs. Indeed, recent analysis of the promoter complex structure of *Mycobacterium tuberculosis* RNAP holoenzyme containing a group 4 σ^{L} factor revealed that the connector region between σ regions 2 and 4 takes the same path as region 3.2 in primary σs, and directly contacts the template DNA strand (Lin, W., Mandal, S., Degen, D., Cho, M., Feng, Y., Das, K. and Ebright, R.H. Structural basis of ECF-sigma-factor-dependent transcription initiation. bioRxiv 381020). Furthermore, structurally unrelated region II.3 occupies the same place and likely performs similar functions in the σ^{54} holoenzyme (42,43). It would therefore be important to test whether mutations in corresponding regions in alternative σ factors may regulate the stability of promoter complexes in a similar way and modulate their sensitivity to the stringent response factors. In the case of eukaryotic RNAP II, a B-reader region of the general transcription factor TFIIB occupies a similar position in the initiating complex (44). In yeast, location of the transcription start point relative to the TATA-box significantly vary in various promoters; thus, the process of template DNA loading into the active site in yeast RNAP II may also be accompanied by DNA scrunching, and the B-reader may play an analogous role to the σ finger in the control of open complex stability.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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