Structural basis of ribosomal RNA transcription regulation

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Ribosomal RNA (rRNA) is most highly expressed in rapidly growing bacteria and is drastically downregulated under stress conditions by the global transcriptional regulator DksA and the alarmone ppGpp. Here, we determined cryo-electron microscopy structures of the Escherichia coli RNA polymerase (RNAP) σ70 holoenzyme during rRNA promoter recognition with and without DksA/ppGpp. RNAP contacts the UP element using dimerized α subunit carboxyl-terminal domains and scrunches the template DNA with the σ finger and β′ lid to select the transcription start site favorable for rapid promoter escape. Promoter binding induces conformational change of σ domain 2 that opens a gate for DNA loading and ejects σ1.1 from the RNAP cleft to facilitate open complex formation. DksA/ppGpp binding also opens the DNA loading gate, which is not coupled to σ1.1 ejection and impedes open complex formation. These results provide a molecular basis for the exceptionally active rRNA transcription and its vulnerability to DksA/ppGpp.
Bacteria sense the availability of nutrition and adjust ribosome biogenesis to optimize their growth. The rate of ribosome biogenesis is primarily determined by rRNA transcription\(^1,2\), which constitutes as much as 70% of total RNA synthesis and is initiated approximately every second from each of the seven rRNA operons (rrnA-E and rrnG-H) in 
\(E.\) coli during exponential growth\(^3\). However, it is drastically repressed under stress conditions such as nutrient-starved stationary phase\(^4\).

rRNA expression is primarily regulated at the initiation stage of RNA synthesis, including RNAP binding to promoter DNA, unwinding the DNA, and escaping from the promoter. The promoters (e.g., \(rrnB\)) for expressing rRNA operons are unique compared with other \(\sigma\)\(^{70}\)-dependent promoters, including (1) the A + T-rich UP element located upstream of the −35 element (from −60 to −40); (2) the G + C-rich discriminator sequence downstream of the −10 element (from −8 to −1); and (3) the transcription start site (TSS) located nine bases downstream from the −10 element (Fig. 1a and Supplementary Fig. 1a). The UP element is recognized by the carboxyl-terminal domain of the \(\alpha\) subunit (\(\alpha\)CTD) and enhances rRNA transcription by more than 30-fold\(^5\). The G + C-rich discriminator and unusual TSS selection of rRNA promoters make its open complex (RPo) unstable, but facilitate RNAP escape from the promoter by reducing abortive RNA cycle prior to the RNA elongation stage\(^6\). These promoter elements play key roles in the wide range of rRNA transcription regulation between nutrient-rich and -poor growth conditions.

rRNA transcription activity is regulated by two small molecules—the initiating ribonucleotide (iNTP) (ATP in the case of \(rrnB\))\(^7\) and the bacterial alarmone ppGpp (guanosine tetraphosphate, aka "magic spot"), which is an allosteric effector of the RNAP-binding global transcription regulator DksA\(^8\)–\(^10\). In the presence of high iNTP concentration, rRNA synthesis starts immediately after RNAP formed the RPo on rRNA promoters, allowing rapid transition to transcription elongation (promoter escape). However, the iNTP-limited condition shifts the equilibrium to favor early intermediates in promoter complex formation, including the closed complex, which is further shifted by DksA/ppGpp binding to RNAP\(^1\). The ppGpp concentration is increased under stress conditions, which enhances DksA-mediated rRNA repression by stabilizing RNAP–DksA complex in a functionally important binding mode\(^11\).

The majority of bacterial RNAP–DNA complex structures determined by X-ray crystallography contain short promoter DNA fragments with a premelted transcription bubble that mimics RPo to maximize its stability required for time-consuming crystallization method\(^12,13\). These studies explained the structural basis of promoter recognition and transcript initiation but left unexplored the interactions of RNAP with duplex DNA around the UP element (via αCTDs) and the

**Fig. 1 Cryo-EM structure of the RNAP-\(rrnB\) closed complex (RPC).** a The sequence of the \(E.\) coli \(rrnB\) promoter DNA used for cryo-EM. The UP element, −35 element, −10 element, transcription start site (TSS, +1) and discriminator sequence are indicated. Alternative TSS from the nonscrunched open complex is indicated by an asterisk. b Orthogonal views of the RPC cryo-EM density map. Subunits and domains of RNAP and DNA are colored and labeled (\(\beta\)prot, \(\beta\)protrusion; tDNA, template DNA; ntDNA, nontemplate DNA). The density of downstream DNA beyond the +4 position is not traceable. Blue lines denote the direction of the DNA axis, with kinks at −37 and −13. The second DNA at the RNAP cleft is indicated (DNA (2nd)). c A magnified view showing the αCTDs and UP element interaction. The domains of \(\alpha\) subunits, \(\sigma\)\(_{4}\), and DNA are depicted as ribbon models with a partially transparent surface. At the top, the sequence of the UP element is shown. The ntDNA (−51 to −48) and tDNA (−54 to −50) sequences binding αCTD and α\(_{2}\)CTD are highlighted in blue and brown, respectively.
contacts with the −10 element (via σ domain 2, residues 92–127 and 373–456 in σ70) in a closed complex and the scrumched DNA bubble in the RPo formed with the rRNA promoters.

Cryo-electron microscopy (cryo-EM) structures of the E. coli RNAP-rpsTp2 promoter complex with a ppGpp-insensitive DksA homolog TraR revealed the RPo formation pathway in the presence of TraR. However, the rpsTp2 promoter for expressing ribosomal protein S20 is distinct from the rrnBP1 promoter that it contains G + C-rich DNA upstream of the −35 element and the TSS 7 bases downstream from the −10 element; therefore, it does not reveal the pathway for rRNA promoter complex formation and the mechanism of rRNA transcription regulation. In addition, the presence of TraR does not allow to infer the unperturbed pathway of the open complex formation by RNAP15–17. Here, we used cryo-EM to visualize the RNAP and rrnBP1 complexes and two additional complexes with DksA/ppGpp on the way to RPo formation.

Results

Cryo-EM structure of the RNAP and rrnBP1 promoter closed complex. To obtain promoter complexes of RNAP with rrnBP1, we preincubated E. coli RNAP σ70 holoenzyme with promoter DNA (Fig. 1a) at 37 °C for 5 min; as a possible way to stabilize the complex, we also added NTPs (ATP and the nonhydrolyzable CTP analog CMPcPP for +1 and +2 NTPs, respectively) prior to cryo-EM grid preparation. In the course of cryo-EM data processing, 3D classification revealed two distinct structures (“Methods” and Supplementary Fig. 2), corresponding to a closed promoter complex (here designated RPCs) and the transcript initiation complex (RPtcs) containing 2-mer RNA. In the RPtcs, the 2-mer RNA transcript (5′-CpA-3′) base-pairs with −1G and +1T in template DNA and is positioned in the post-translocated conformation. The detailed RPtcs structure will be described in a separate report.

We determined the RNAP-rrnBP1 RPC structure with an overall resolution of 4.14 Å (Supplementary Table 1). The cryo-EM density shows that RNAP binds the duplex DNA from −60 to +3, which remains fully double-stranded (Fig. 1b, Supplementary Fig. 4 and Supplementary Movie 1), but the density of downstream DNA beyond position +4 is not traceable. Instead, a second DNA binds to the RNAP cleft due to ejection of σ1 from the RNAP cleft during RPc formation as described later.

The cryo-EM density for both αCTDs (residues 248–329), the linkers (residues 236–247) connecting to αNCTDs (residues 1–235), and the UP element DNA were traceable in the RPC, allowing us to investigate how each αCTD binds to the UP element unambiguously (Fig. 1c and Supplementary Movie 1). Two αCTDs form a head-to-tail dimer and bind DNA side-by-side in the middle of the UP element (−51 to −48 on non-template DNA (ntDNA) and −54 to −50 on template DNA (tDNA)), which is in good agreement with the DNA footprinting results. Although α subunits form a homodimer, two α subunits play different roles in RNAP, with one (α1) adjacent to the β subunit and the other (α2) adjacent to the β′. Compared to the αCTD, the αCTD is positioned proximally to the −35 element, which explains the result of DNA cleavage by hydroxyl radicals from chelated Fe attached at each of the two αCTDs. The αCTDs bind DNA with a narrow minor groove, which is formed due to the presence of an A/T stretch sequence, as revealed by the recent X-ray crystallographic study of the αCTD and UP element interaction. The side chains of R265 and N294 from both αCTDs are inserted into the DNA minor groove, and basic residues (K291 and K298) are involved in salt bridges with the DNA phosphate backbone (Supplementary Fig. 5a). The linkers of both α subunits are fully extended, and slight DNA bending centered at the −37 position is required for the αCTDs binding to the UP element (Fig. 1c). Consistent with this observation, shortening of the linkers by only three amino acids reduces rrnBP1 transcription. Several studies have proposed that distant upstream DNA (near the −100 position) warps around RNAP on the RPo formation pathway, and the interaction of αCTDs and the UP element is one of the major driving forces for this DNA wrapping. However, αCTDs do not bend the DNA around their binding site in the RPC structure determined in this study. This observation suggests that the contacts of αCTDs with the UP element by themselves are not sufficient for wrapping of upstream DNA around RNAP. This may possibly result from the destabilization of upstream DNA and RNAP interactions by truncation of the promoter fragment at the −60 position and/or from specific conditions used for the cryo-EM sample preparation.

The position and orientation of αCTDs in the rrnBP1 RPC structure are distinct from those of αCTDs in the RNAP complex with the rpsTp2 promoter, lacking the UP element (PDB: 6PSQ), which binds DNA just upstream of the σ domain. (α2) bound to the −35 element (Supplementary Fig. 3b). This indicates that the mode of αCTDs interactions with upstream DNA can be significantly different in various promoters depending on the presence of the UP element.

The RPC structure shows how σ1 binds the duplex form of the −10 element. The DNA encoding the −10 element is anchored by σ domain 2 and slightly bends around the upstream edge of the −10 element, allowing the downstream part beyond the −10 element to reach the other side of the RNAP cleft comprising the β protrusion domain (Fig. 1b and Supplementary Movie 1). The σ region 2.3 (σ2.3, residues 417–434) contacts the −10 element by fitting into the DNA major groove seemingly without sequence-specific interaction, indicating that σ2.3 recognizes the shape and/or curvature around the −10 element. This finding is in agreement with the previous proposal that σ70 does not contact the −10 element DNA bases when it is in duplex form.

Cryo-EM structure of the RNAP and rrnBP1 promoter open complex (RPO). To obtain the structure of the open RNAP-rrnBP1 complex (RPO) with melted DNA bubble, we preincubated RNAP holoenzyme and promoter DNA at 37 °C for 5 min prior to cryo-EM grid preparation (Supplementary Fig. 3). 3D classification revealed one major class of particles corresponding to RPO, and its structure was determined with an overall resolution of 3.5 Å. In comparison with the RPC, the RPO structure shows significant differences in the UP element (from −60 to −40), the downstream DNA (from −14 to +20) and the conformation of the σ factor. The cryo-EM density for RPO covers DNA from −44 to +20, including an open bubble from −13 to +2 and the downstream DNA accommodated in the RNAP cleft (Fig. 2a, Supplementary Fig. 4b, e, and Supplementary Movie 2). In contrast to the RPC, αCTDs and UP elements are disordered.

Basic residues in the σlobe (K163, K169, K191, R202, and K203), β′jaw (K1151, K1167, K1170, K1172, and R1174) and βclamp (R133, K213, K216, K219, and R311) participate in the interaction with downstream DNA to stabilize the RPo (Fig. 2b). The importance of these interactions in rRNA transcription regulation is supported by the isolation of ΔΔksa suppressor mutations in these domains. The βgate loop (βGL, residues 368–378) in the σlobe domain contacts the T94, R99, and R103 residues of σ region 1.2 (σ1.2, residues 92–127) to enclose the RNA cleft (Fig. 2b). The βGL, σ1.2, and σ2.1 (residues 373–396) contact the ntDNA strand of the discriminator from positions −8 to −6 (Fig. 2c and Supplementary Movie 3); consistently, the βGL deletion and substitutions in σ1.2 destabilize the RPo.
The RNAP and rrnB1 complex starts RNA synthesis at the position 9 bp downstream from the −10 element (+1A), which requires DNA scrunching29. The RPo structure revealed the path of the scrunched template strand, in which the G-7 base of tDNA fits into a pocket surrounded by the βlid, σfinger and C-6 base. β and σ are depicted as ribbon models with transparent surfaces, and DNA is shown as a stick model and CPK representation. The residues forming salt bridges and Van der Waals interactions with the G-7 base are shown (depicted by red and black dashed lines).

Fig. 2 Cryo-EM structure of the RNAP-rrnB1 open complex (RPo). a Orthogonal views of the RPo cryo-EM density map. Subunits and domains of RNAP and DNA are colored and labeled the same as in Fig. 1b. b The structure of the RPo, highlighting basic residues of the βlobe/Si1 (blue), βjaw/Si3 (purple), and βclamp (pink) interacting with downstream DNA (green) to stabilize the RPo. The structure is shown as a ribbon model with a transparent surface, and the basic residues are shown as spheres and labeled. Close-up view of RNAP (βGL, σ1.1, and σ1.2) and discriminator DNA (ntDNA) interaction. β and σ are depicted as ribbon models with transparent surfaces, and DNA is shown as CPK spheres. The G-8, C-7, and G-6 bases (stick model with transparent CPK spheres) that form salt bridges and Van del Waals interactions with residues from the βGL and σ1.2 (side chains shown as sticks; βGL R371 and D374; σ1.2 R99 and M102) are shown (depicted by red and black dashed lines). d Close-up view of the RNAP (βlid and σfinger) and discriminator DNA (tDNA) interaction. The G-7 base inserts into the pocket formed by the βlid, σfinger and C-6 base. β and σ are depicted as ribbon models with transparent surfaces, and DNA is shown as a stick model and CPK representation. The residues forming salt bridges and Van del Waals interactions with the G-7 base are shown (depicted by red and black dashed lines). e Comparison of the σfinger in RPo-rrnB1 (this study, orange) and RPo-rpsT214 (gray). The RPo-rrnB1 structure is depicted as a cartoon (RNAP) and stick (DNA) models. When the rrnB1 tDNA scrunches at the −7G(t) position, −5G(t) is located below the σfinger (orange), which shifts the σfinger position compared to that in nonscrunched RPo (gray). The σfinger dislocation (black arrow, 5 Å at E515) makes additional space for RNA extension (red arrow).
stranded junction at the RNAP active site (from −2 to +2 positions) disordered.

Open complex scRUNch may facilitate promoter escape of RNAP by reducing abortive RNA cycle\(^{29,31}\). Compared with RPo-
rrpTP2 containing nonscRUNched tDNA\(^{14}\), RPo-rrnBP1 shifts the of\(\text{finger} \sim 5\) Å away from tDNA, allowing accommodation of one additional base of RNA before its 5′-end reaches the of\(\text{finger}\) (Fig. 2e). Since the of\(\text{finger}\) is one of the major obstacles to promoter escape\(^{32-34}\), the partially displaced of\(\text{finger}\) in the RPo may reduce the abortive RNA cycle or may prevent the formation of inactive mori\(\text{bund}\) complexes\(^{31,35}\), promoting the robust expression of rRNA.

Cryo-EM structures of the RNAP and rrnBP1 promoter complex with DksA/ppGpp (RP-DksA/ppGpp). To reveal how DksA/ppGpp binding to RNAP downregulates rRNA transcription, we visualized the RNAP, rrnBP1, and DksA/ppGpp complex (RP-DksA/ppGpp) by cryo-EM (Supplementary Table 1 and Supplementary Fig. 6). The classification of the cryo-EM data gave rise to two structures that differed mainly within the RNAP cleft: the first class shows the globular density corresponding to \(\sigma_{1,1}\) (class I, RP1-DksA/ppGpp), and the second class shows the helical density corresponding to the downstream DNA (class II, RP2-DksA/ppGpp) (Fig. 4a, b and Supplementary Movie 4). In addition, the positions of \(\beta_{\text{lobe}}/\text{Si1}\) are different in these classes.

In both classes, the cryo-EM density maps show ppGpp binding at sites 1 and 2 and DksA binding at the RNAP secondary channel (Supplementary Fig. 6e), as observed in the previous X-ray crystallography study\(^{11}\). DksA binds RNAP with its globular domain (G domain, contacts with the \(\beta\)rim helix), coiled-coil tip (CC tip, contacts with the active site), CC (contacts with the bridge helix, the trigger loop, and linkers connecting to the \(\beta\)Si3), and the C-terminal \(\alpha\) helix (CT-helix, contacts with the \(\beta\)lobe/Si1 domain) (Fig. 4a and Supplementary Movie 4). The CC of DksA prevents trigger helix formation and blocks NTP entry from the secondary channel, indicating that DksA must be displaced before RNAP initiates RNA synthesis\(^{11,36}\).

Both classes show the duplex DNA density from positions −42 to −14 (from the downstream edge of the UP element to the upstream edge of the −10 element) and also show the ssDNA density of the non\(\text{template}\) strand of the −10 element (Supplementary Fig. 4). RP1-DksA/ppGpp retains \(\sigma_{1,1}\) in the RNAP cleft, indicating that it represents an early stage intermediate during the closed to open complexes transition. While the transcription bubble is likely partially open in RP1-DksA/ppGpp, the density of ntDNA from −5 to +20 and of tDNA from −13 to +20 is not traceable. Analysis of RP1-DksA/ppGpp reveals a DksA/ppGpp-induced conformational change in \(\beta_{\text{lobe}}/\text{Si1}, \beta_{\text{jaw}}/\text{Si3}\) and \(\beta\)clamp, opening the downstream DNA cleft in RNAP and likely reducing the stability of RPo (Fig. 4d). The interactions of the \(\beta_{\text{lobe}}/\text{Si1}\) domain with DksA CT-helix and the conformational change of Si1 were previously observed in the crystal structure of the RNAP-DksA/ppGpp complex\(^{11}\) but were smaller than in the cryo-EM structure, likely because of the crystal packing.
The conformational change in βlobe/Si1 establishes a new contact with the DksA CT-helix, which is only observed in the cryo-EM structure (Fig. 4c and Supplementary Movie 4); the deletion of βSi1 was shown to reduce the DksA affinity to RNAP and impair its function 37. Alanine substitution of an aspartate residue in the CT-helix directly involved in this interaction (D137A) decreases rrnB P1 inhibition by DksA both in the absence and in the presence of ppGpp (Table 1).

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Fig. 4 Cryo-EM structures of the RNAP-rrnB P1 complex with DksA/ppGpp (RP-DksA/ppGpp). a Orthogonal views of the RP1-DksA/ppGpp cryo-EM density map. DNA, RNAP and DksA (G, G domain; CC, CC domain; CT, CT-helix) are indicated and colored. σ1,1 is highlighted by a white dash. b The RP2-DksA/ppGpp cryo-EM density map. The downstream DNA is accommodated in the RNAP cleft. c Close-up view of the βlobe/Si1 conformational changes upon DksA binding, σ1,1 ejection and downstream DNA binding. The structures of the βlobe/Si1 in RP1-DksA/ppGpp (light blue), RP2-DksA/ppGpp (white), and RPo (black) are depicted as ribbon models with transparent surfaces and ribbon models (DksA, BH: bridge helix) of RP1-DksA/ppGpp. The interaction between βlobe/Si1 and DksA in RP1-DksA/ppGpp is highlighted by a black dashed oval. d Conformational changes in the RNAP mobile domains upon binding of DksA/ppGpp. The structures of the RNAP mobile domains in RP1-DksA/ppGpp (colored) and RPo (black) are depicted as ribbon models with transparent surfaces of RP1-DksA/ppGpp.

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Ejection of σ1,1 and conformational changes in σ domain 2 during a RPo formation. The RPo structure revealed the ejection of σ1,1 from the RNAP cleft and significant conformational changes in σ domain 2, including σ1,2 and the nonconserved region between regions 1 and 2 (σNCR, residues 128–372), in comparison with the apo-form holoenzyme RNAP 38 and the RPo containing rrnB P1 (this study).

From the apo-form to RPo, σ1,2/σNCR of RNAP holoenzyme undergo a rigid rotation toward the clamp to establish contact with the βclamp-toe (βCT, residues 143 to 180) (RPc, Fig. 5a). Although this interaction was not observed in any previous structural study, it was predicted based on the biochemical/genetic analysis of RNAP promoter escape and early elongation...
It was shown that the interaction of the \( \sigma_{NCR} \) and \( \beta' \text{CT} \) is important for promoter escape and hinders early elongation pausing, and amino acid substitutions at the interface modulate both processes (Supplementary Fig. 7).

The \( \beta\text{GL} \) contacts the N-terminus of \( \sigma_{1.2} \) to enclose the RNAP cleft in the apo-form RNAP, which prevents DNA loading (apo, Fig. 5a), but the same interaction in the RPo stabilizes the open complex bubble (Fig. 2b). In the case of RPC, the \( \sigma_{1.2}/\sigma_{NCR} \) rotation disrupts the \( \beta\text{GL} \) and \( \sigma \) contact and widens the gap that allows the ejection of \( \sigma_{1.1} \) and the entrance of discriminator DNA for the open complex bubble formation (RPC, Fig. 5a). Compared with the apo-form RNAP, the \( \sigma_{NCR} \) and \( \beta' \text{CT} \) interaction in the RPo closes the \( \beta' \text{clamp} \), which likely stimulates the ejection and prevents re-entry of \( \sigma_{1.1} \) due to its steric clash with the \( \beta' \) clamp (Fig. 5b).

\( \sigma_{NCR} \) contains a highly negatively charged region (acidic loop, residues 167–213) (Supplementary Fig. 8a). Its conformation has not been determined due to its dynamic behavior, but since it is located near \( \sigma_{2.3} \), it seems to prevent nonspecific DNA binding to \( \sigma_{2.3} \) (Supplementary Fig. 8b). We speculate that after RNAP recognizes the UP and \( -35 \) elements, loading of the \( -10 \) element DNA onto \( \sigma \) domain 2 triggers \( \sigma_{NCR} \) rotation due to charge-charge repulsion. After DNA unwinds around the \( -10 \) element, \( \sigma_{NCR} \) returns to its position, as seen in the RPo akin to the apo-form RNAP, and may enhance the electrostatic interaction between \( \sigma_2 \) and \( -10 \) element DNA (Supplementary Fig. 8c).

Consistently, deletion of the acidic loop (\( \Delta\sigma_{AL} \)) had a weak destabilizing effect on the \( \text{rrnB} P_1 \)-RNAP complex, without strong effects on DksA inhibition (Fig. 3 and Table 1).

| RNAP        | \( K_{d,app} (nM) \) |
|-------------|----------------------|
| WT          | 2200 ± 430           |
| \( \beta'D256A \) | 1450 ± 150     |
| \( \Delta\sigma_{AL} \) | 3000         |
| WT RNAP +  | 3900                 |
| DksA D137A  | 3000                 |

The apparent dissociation constants for DksA binding to promoter complexes (\( K_{d,app} \)) were calculated from the efficiency of transcription inhibition in titration experiments. The numbers in below \( K_{d,app} \) values indicate fold changes in \( K_{d,app} \) relative to the wild-type RNAP.

**Table 1** Apparent affinities of DksA to wild-type and mutant RNAPs on the \( \text{rrnB}P_1 \) promoter.

**Fig. 5 Opening the DNA loading gate by moving the \( \sigma_{1.2}/\sigma_{NCR} \) or \( \text{lobe}/\text{Si1} \) domain.**

a Comparison of the \( \sigma_{1.2}/\sigma_{NCR} \) and \( \text{lobe}/\text{Si1} \) conformations in apo-RNAP (middle), RPC (left), and RNAP-DksA/ppGpp (R-DksA, right). DNA is removed from the RPI-DksA/ppGpp to model this complex. RNAP (subunits and domains), DksA and DNA are indicated. Close-up views of the RNAP cleft are shown below. The DNA loading gate is closed in the apo-RNAP due to the \( \beta \text{gate loop} \) (\( \beta\text{GL} \)) contacts with \( \sigma_{1.1}/\sigma_{1.2} \) (white dashed oval). The opening of the DNA loading gate in RPC and RPI-DksA/ppGpp is indicated by blue and red arrows, respectively.

b A proposed model of \( \sigma_{1.1} \) ejection in the RPC. The RPC is depicted as a transparent surface with cartoon models of the clamp (purple) and the \( \text{lobe}/\text{Si1} \) (blue). The clamp in the apo-form RNAP and the \( \text{lobe}/\text{Si1} \) in RPI-DksA/ppGpp are colored gray and white, respectively. In the RPC, the \( \sigma_{NCR} \) rotation (black arrow) contacts the \( \beta'\text{CT} \), resulting in clamp movement toward \( \sigma_{1.1} \) (red arrow) and a steric clash with \( \sigma_{1.1} \) (white oval).
σ₁,₂/ΔσNCr changes. First, the presence of σ₁,₁ at the RNAP cleft in the apo-holoenzyme prevents any DNA binding at this cleft; therefore, σ₁,₁ ejection has to be completed prior to binding of DNA and it requires disruption of the βG domain and σ contacts coupled to the σNCr rotation. Second, none of the available promoter complex cryo-EM structures containing double-stranded DNA at the RNAP cleft show the σNCr rotation suggesting that the downstream DNA binding by itself is unlikely to trigger the conformational change in σ₁,₂/ΔσNCr. Thus, the RPC structure illustrates how the downstream DNA cleft in RNAP becomes prepared for binding of the downstream DNA duplex during open complex formation.

DksA/ppGpp binding to RNAP also partially opens the DNA loading gate by moving the βlobe/Si1 away from σ₁,₁/σ₁,₂, but it is not strictly coupled to the σ₁,₁ ejection from the RNAP cleft (R-DksA, Fig. 5a). Similarly, the structures of the RNAP–TraR complex and several RNAP–DNA complex intermediates prepared in the presence of TraR also showed the opening of the DNA loading gate by shifting the βlobe/Si1 position but did not show σ₁,₂/ΔσNCr rotation. Furthermore, σ₁,₁ was not ejected from the RNAP cleft at the stage of RPC formation (Supplementary Fig. 9a).  

To understand the role of σ₁,₁ in rRNA transcription, we characterized an RNAP derivative lacking σ₁,₁ (Δσ₁,₁–RNAP) in terms of its rrnB1P transcription activity and sensitivity to DksA. Compared to the wild-type (WT) RNAP, Δσ₁,₁–RNAP has an increased rrnB1P complex stability, both in the absence of DksA (increase in t₁,₂ from 34 s to 115 s) and in its presence (increase in t₁,₂ from <10 s to 20 s) (Fig. 5a, b), and decreased sensitivity to DksA (Table 1). At the same time, the Δσ₁,₁ deletion (and, similarly, Δσ₁,₂) does not change the transcription start site in rrnB1P suggesting that σ₁,₁ does not affect DNA scrunching during transcription initiation (Fig. 3c). Overall, the results indicate that σ₁,₁ plays an important role in the destabilization of rRNA promoter complexes and their regulation by DksA/ppGpp.

**Discussion**

Mechanism of rRNA-specific transcription inhibition by DksA/ppGpp. Structural and biochemical studies of bacterial RNAP transcription suggest that the order of DNA loading around the TSS and DNA opening may be interchangeable during promoter recognition (i.e., DNA melts first outside RNAP (melt-load) or DNA melts after loading inside the RNAP cleft (load-melt)) depending on σ factors, promoters, transcription factors and conditions. By combining structural and biochemical data from this and previous studies, we propose two pathways of RPo formation (Fig. 6 and Supplementary Movie 5). We hypothesize that RNAP may use alternative mechanisms of RPo formation with rrnB1P and possibly other promoters, requiring the opening of the DNA loading gate (disrupting the βG domain contact to σ), σ₁,₁ ejection from the DNA binding channel, and unwinding of the −10 element plus discriminator DNA, depending on the absence or presence of DksA/ppGpp.

Without DksA/ppGpp (top, RPo formation), free RNAP (R) binds promoter DNA (RPc), which opens the DNA loading gate by ejecting σ₁,₁ from the RNAP cleft, making RNAP competent for melting and loading discriminator DNA (RPi) into the RNAP cleft, which results in efficient RPo formation. The scrunched open complex (RPo) releases RNAP from the rRNA promoter rapidly to proceed with RNA synthesis (EC).

In the presence of DksA/ppGpp (bottom, RPo formation with DksA/ppGpp), DksA/ppGpp binding to RNAP rotates the βlobe/ΔσSi to DksA, which partially opens the DNA loading gate by disrupting the interaction between GL and σ (R-DksA). However, σ₁,₁ ejection is uncoupled from RPo formation (RPc–DksA), and σ₁,₁ can remain inside the RNAP cleft until the late stages of the open complex formation (RPi–DksA). This pathway favors the melt-load model for RPo formation (RPo–DksA), in which DNA is accommodated above the βlobe domain and unwinds outside the RNAP cleft (Supplementary Fig. 9b) followed by single-stranded tDNA entry into the active site of RNAP. DNA unwinding outside the RNAP cleft is unfavorable in the DksA/ppGpp-free RNAP due to a steric clash of the discriminator DNA with the βlobe. The progression of DNA unwinding from the −10 element to the TSS is energetically less favorable for DksA/ppGpp-sensitive promoters (e.g., rrnB1P and rpsT2P) containing the G+C-rich discriminator than for less DksA/ppGpp-sensitive promoters (e.g., T7A1 and RNA1) containing an A+T rich discriminator (Supplementary Fig. 10).

**Methods**

**Preparation of rrnB1P DNA.** The rrnB1P promoter DNA was synthesized (IDT) according to the native rrnB1P sequence and annealed in a 40 μl reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM EDTA to a final concentration of 0.5 mM. The solution was heated at 95 °C for 10 min, and then the temperature was gradually decreased to 22 °C. The sequence of the nontemplate strand is 5′-CATGAAATTTTTTTATTTTCCTGTTCTGACGCGGAAATA ACCTCTCTATAATGGCCGACCGCTAGACAAGGCTCTACGAG-3′. The transcription start site is underlined, and the template sequence is 5′-CTCTAGAAGCT CGTTGCGTGTGGGGCATATTAGGAAGGTATTTCCGCCCTGACAAAGG AAATTTAATATATTCTGT-3′.
Cryo-EM sample preparation. *E. coli* σ70 RNAP holoenzyme and DksA were purified, as described previously11,50. To prepare the RNAP and *rrnB*P1 promoter complex, *E. coli* σ70 RNAP (20 μM) and *rrnB*P1 promoter DNA (40 μM) were preincubated for 5 min at 37 °C in buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT and 5 mM MgCl2). This sample was used for determining the cryo-EM structure of RPo (Supplementary Fig. 3). To stabilize the RPo, NTPs (ATP and a nonhydrolyzable CMPCPP (cytidine-5′-[α,β]-methylene)triphosphate, Jena Bioscience) (2 mM each) were added to the RNAP- *rrnB*P1 promoter complex; however, this condition formed the RPc and RPtic (Supplementary Fig. 2) as described in “Results”.

To prepare RP-DksA/ppGpp (Supplementary Fig. 6), *E. coli* σ70 RNAP (20 μM) was preincubated with a fivefold molar excess of DksA (100 μM) and ppGpp (2 mM) for 5 min at 37 °C in buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 5 mM MgCl2). The *rrnB*P1 promoter DNA (40 μM) was added to the reaction and further incubated for 5 mins at 37 °C. Before freezing the grids, 8 mM CHAPSO (Hampton research) was added to the reaction. A 3.5 μL sample was applied to a glow-discharged C-Flat Holey Carbon grid (Cu 2/1, 400 mesh), blotted and plunge-frozen in liquid ethane using a Vitrobot Mark IV (FEI, USA) with 95% humidity at 4 °C.

Cryo-EM data acquisition. The grid was imaged using a 300 kV Titan Krios (Thermo Fisher) microscope equipped with a K3 direct electron detector (Gatan) and controlled by the Latitude S (Gatan, Inc.) software at the National Cancer Institute’s Cryo-EM Facility at Frederick. The defocus range was −1.0 to −3.0 μm, and the magnification was ×81,000 in electron counting mode (pixel size = 1.08 Å/pixel). Forty frames per movie were collected with a dose of 1.125 e−/Å²/frame, giving a total dose of 45 e−/Å².

Cryo-EM data processing. The RNAP-*rrnB*P1 complex with ATP/CMPCPP data was processed using Relion3.0.8. A total of 8315 movies were collected, aligned and dose weighted using MotionCor2. CTF fitting was performed with Gctf. Approximately 1000 particles were manually picked to generate particle templates followed by automated picking, resulting in a total of 1,449,010 particles subjected to 2D classification. From the 2D classes, 1,442,810 particles were chosen for the 3D classification to four classes. Poorly populated classes were removed, resulting in datasets of 541,257 (37%) particles for the first class (RPc) and 464,512 (32%) particles for the second class. The first class was further 3D classified without alignments twice to further clean the data, resulting in datasets of 67,187 particles. The particles were reﬁned and postprocessed to generate the density map at 4.14 Å resolution. The resolution of the density map of the second class was 3.53 Å.

Cryo-EM data processing. The RNAP-*rrnB*P1 complex with ATP/CMPCPP data was processed using Relion3.0.8. A total of 8315 movies were collected, aligned and dose weighted using MotionCor2. CTF fitting was performed with Gctf. Approximately 1000 particles were manually picked to generate particle templates followed by automated picking, resulting in a total of 4748 movies collected, aligned and dose weighted using MotionCor2. CTF fitting was performed with Gctf. Approximately 1000 particles were manually picked to generate particle templates followed by automated picking, resulting in a...
total of 563,500 particles. Particles were 2D classified, and 561,753 particles were chosen for the 3D classification. Of the four 3D classes, class 1 (RPo) was the most populated class (549,752 particles, 62%) and was autorefined. The map was postprocessed to give a structure of RPo at 3.5 Å.

The RP-DksA/ppGpp complex data were processed using cryoSPARC V2.9.0. A total of 4,926 movies were collected, and the movies were aligned, and dose weighted using Patch-motion correction. CTF fitting was performed with Patch-CTF estimation. Initially, ~1000 particles were manually picked to generate particle templates followed by automated picking, resulting in a total of 418,049 particles subjected to 2D classification. After two rounds of 2D classification to remove junk particles, 361,048 particles were used to generate two ab initio models. Junk particles were removed, resulting in a dataset of 275,629 particles for the 3D classification (heterogeneous refinement). Poorly populated classes were removed, resulting in a dataset of 49,995 particles to generate the density map at 3.62 Å resolution for the first class (RPI-DksA/ppGpp) and a dataset of 79,275 particles to generate the density map at 3.58 Å for the second class (RP2-DksA/ppGpp). The particles were 3D auto-refined without the mask and postprocessed (homogeneous refinement).

Structure refinement and analysis. To refine the closed and open complex structures, the E. coli RNAP holoenzyme crystal structure (PDB: 4YG2) was manually fit into the cryo-EM density map using Chimera58 and real-space refined using Phenix56. In the real-space refinement, the domains of RNAP were rigid-body refined and then subsequently refined with secondary structure, Ramachandran, rotamer, and reference model restraints. To refine the structures of RPI-DksA/ppGpp and RP2-DksA/ppGpp, the RNAP crystal structures (PDB: 5SV5) were manually fit into the cryo-EM density map using Chimera. DNA was manually built using Coot57. The structure was refined by the same method as the closed and open complex structures. To superpose RNAP, we used the nDts and the catalytic domains (DPBR domains from the β and β′ subunits) of RNAP as references. Figures were prepared by Chimerax58 and PymOL.

Preparation of RNAP and transcription factors for in vitro transcription. Mutant variants of RNAP, σ70, and DksA were obtained by site-directed mutagenesis. The sequences of all primers used in this study are shown in Supplementary Table 2. The D256A substitution in the β′ subunit was obtained in pVS10 encoding all RNAP subunits, with the σ70 and DksA variants containing an N-terminal His6-tag were cloned into pET28. To obtain ΔHis-tag, the 5′-terminal His-tag was cloned into pET28. To obtain Δα, the 5′-terminal part of the rpoD gene encoding residues 2–94 was deleted. To obtain Δα+β′, codons 168–212 were replaced with three glycine codons. All proteins were expressed in E. coli BL21(DE3). The wild-type and mutant core RNAPs were purified using Polymin P precipitation followed by heparin (HiTrap Heparin column), Ni-affinity (HiTrap HP column), and anion exchange (MonoQ column) chromatography steps (all columns from GE Health-care)59. The wild-type and mutant σ70 variants were purified from inclusion bodies with subsequent renaturation and Ni-affinity chromatography60. The Δα+β′ protein was subjected to thrombin protease (GE Health-care) treatment in PBS buffer (10 h of incubation with 10 μg/ml thrombin) to remove the N-terminal His-tag and His-tagged threonin.

To purify DksA, bacterial pellet from 0.5 liters of cell culture was resuspended at 25 ml of lysis buffer (50 mM Tris-HCl, pH 7.9, 200 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, and 1 mM DTT) and lysed using a French press. The supernatant obtained after centrifugation was loaded onto a 5-ml HiTrap chelating column (GE Healthcare) charged with Ni2+ and equilibrated with loading buffer (10 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.5 mM 2-mercaptoethanol, 0.1 mM ZnCl2). The column was washed with the same buffer containing 60 mM imidazole, and DksA was eluted with buffer containing 300 mM imidazole and dialyzed overnight against 50 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, and 0.1 mM ZnCl2. Glycerol was added to 50%, and aliquots were stored at −70 °C.

Transcription in vitro. Analysis of transcription in vitro was performed using a supercoiled pTZ191 template containing rnpB1 cloned 88 nt upstream of the his terminator; the second transcript monitored in the assays was 110 nt RNAI. For measurements of promoter complexes, 50 μM of σ70 and DksA variants containing an N-terminal His6-tag were cloned into a 1.1 protein expression vector (pET28), followed bydireccion with Ni-NTA agarose (GE Health-care) to remove the His-tag and His-tagged threonin.

To purify DksA, bacterial pellet from 0.5 liters of cell culture was resuspended at 25 ml of lysis buffer (50 mM Tris-HCl, pH 7.9, 200 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, and 1 mM DTT) and lysed using a French press. The supernatant obtained after centrifugation was loaded onto a 5-ml HiTrap chelating column (GE Healthcare) charged with Ni2+ and equilibrated with loading buffer (10 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.5 mM 2-mercaptoethanol, 0.1 mM ZnCl2). The column was washed with the same buffer containing 60 mM imidazole, and DksA was eluted with buffer containing 300 mM imidazole and dialyzed overnight against 50 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, and 0.1 mM ZnCl2. Glycerol was added to 50%, and aliquots were stored at −70 °C.

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**Author contributions**
Y.S. prepared samples and cryo-EM grids. Y.S. and M.Z.Q. collected and processed cryo-EM data. K.S.M. built, refined, and validated the structures. D.P. and D.E. performed biochemical assays under the supervision of A.K. K.S.M. designed and supervised research. All authors participated in the interpretation of the results and in writing the paper.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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