Allosteric control of *Escherichia coli* rRNA promoter complexes by DksA

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The *Escherichia coli* DksA protein inserts into the RNA polymerase (RNAP) secondary channel, modifying the transcription initiation complex so that promoters with specific kinetic characteristics are regulated by changes in the concentrations of ppGpp and NTPs. We used footprinting assays to determine the specific kinetic intermediate, RP_I, on which DksA acts. Genetic approaches identified substitutions in the RNAP switch regions, bridge helix, and trigger loop that mimicked, reduced, or enhanced DksA function on rRNA promoters. Our results indicate that DksA binding in the secondary channel of RP_I disrupts interactions with promoter DNA at least 25 Å away, between positions −6 and +6 (the transcription start site is +1). We propose a working model in which the trigger loop and bridge helix transmit effects of DksA to the switch region(s), allosterically affecting switch residues that control clamp opening/closing and/or that interact directly with promoter DNA. DksA thus inhibits the transition to RP_I. Our results illustrate in mechanistic terms how transcription factors can regulate initiation promoter-specifically without interacting directly with DNA.

[*Keywords*: RNA polymerase; promoter; DksA; ppGpp; transcription initiation; ribosome synthesis]

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DksA, ppGpp, and NTPs work together to regulate rRNA synthesis in *Escherichia coli* (Paul et al. 2004). DksA concentrations are relatively constant (Rutherford et al. 2007), but ppGpp and NTP concentrations vary dramatically with nutrient availability (Murray et al. 2003). Inactivation of the *dksA* gene derepresses rRNA transcription, uncoupling ribosome production from the cellular demand for protein synthesis, because direct modification of RNA polymerase (RNAP) by DksA is needed for changes in the concentrations of ppGpp and NTPs to exert effects on the transcription initiation complex (Paul et al. 2004).

The mechanism of DksA action remains unclear. Unlike conventional regulators of transcription initiation, DksA does not bind to DNA but instead interacts directly with RNAP (Paul et al. 2004; Perederina et al. 2004). Biochemical studies and structural similarities between DksA and the transcription elongation factors GreA and GreB suggest that DksA binds in the RNAP secondary channel (Opalka et al. 2003; Perederina et al. 2004; S.T. Rutherford, I. Toulokhonov, C.E. Vrentas, W. Ross, and R.L. Gourse, unpubl.), but there is no structure of DksA bound to RNAP, and the precise interactions between RNAP and DksA have yet to be defined.

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Because DksA binds RNAP instead of a specific DNA sequence, it has the potential to affect all promoter complexes. Consistent with this prediction, DksA decreases the lifetimes of complexes formed by all promoters tested to date (Paul et al. 2004, 2005; Rutherford et al. 2007). However, DksA directly affects transcriptional output only from a subset of promoters, including many needed for the synthesis of ribosomes, virulence, membrane stress responses, and amino acid biosynthesis and transport (for review, see Haugen et al. 2008). The promoter-specific effects of DksA on transcriptional output are dictated by the intrinsic kinetic properties of different promoters (Haugen et al. 2008).

Transcription begins with binding of RNAP holoenzyme (subunit composition $\alpha_2\beta\beta'\omega\sigma$) to the promoter, forming an initial complex in which the DNA strands remain paired (closed complex, RP_C) (Fig. 1A). This complex undergoes a series of conformational changes in both the DNA and RNAP that ultimately result in formation of a transcriptionally competent open complex (RP_O) in which the DNA strands are separated from approximately −11 to approximately +3 (Haugen et al. 2008 and references within). At least one additional kinetically significant intermediate (referred to here as RP_I) has been identified between RP_C and RP_O (Fig. 1A; Saecker et al. 2002; for review, see Haugen et al. 2008). Transcription initiation likely proceeds through the same series of intermediates at all promoters, but the fractional occupancy of these intermediates at equilibrium differs at different promoters.

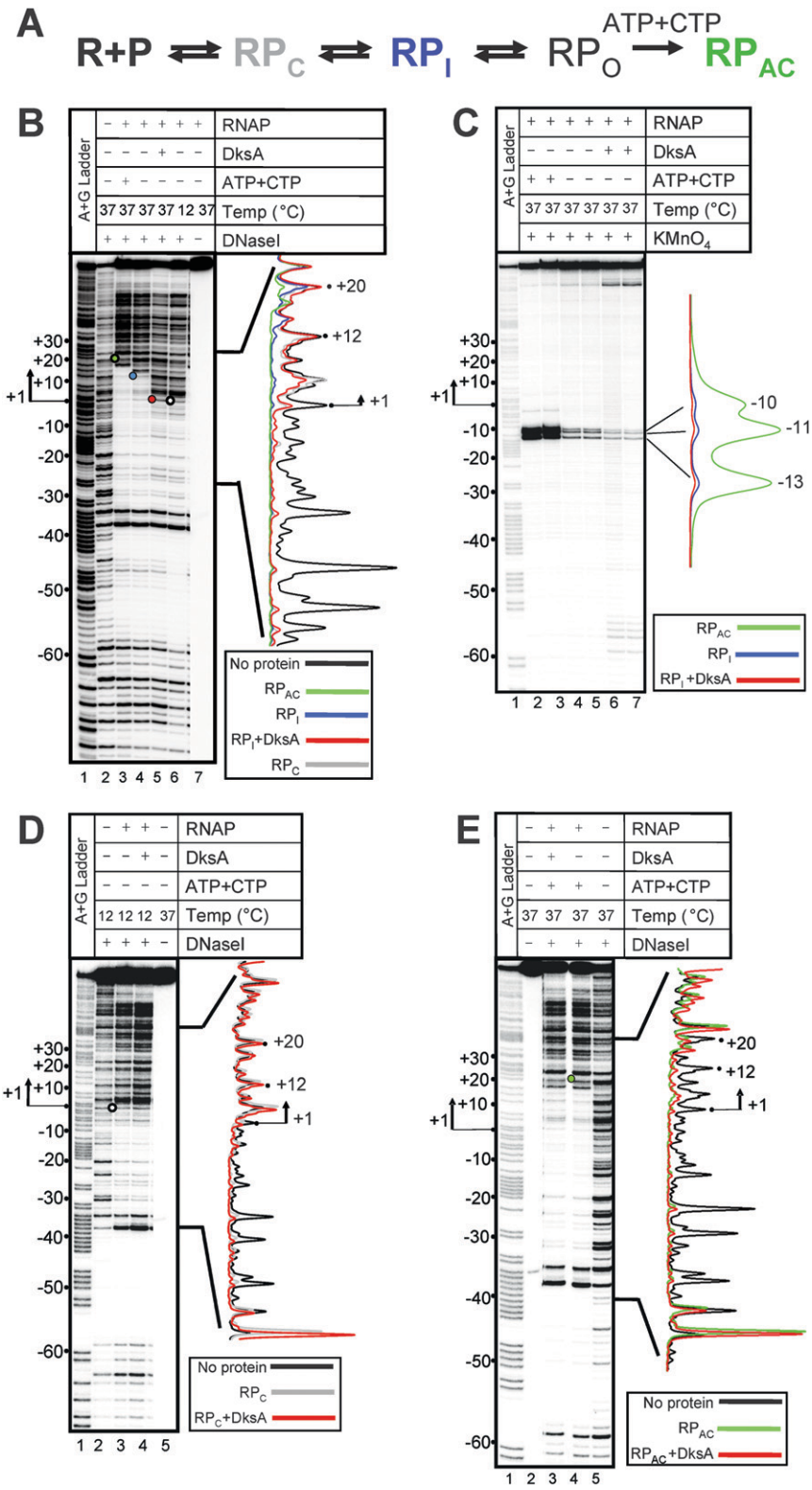


Figure 1. DksA shifts RP_I at *rrnB* P1 to RP_C. (*A*) Kinetic steps in transcription initiation at *rrnB* P1. (R) RNAP; (P) promoter; (RP_C) closed complex; (RP_I) intermediate complex; (RP_O) open complex (transiently occupied at *rrnB* P1); (RP_{AC}) complex formed with ATP and CTP. (*B*,*D*,*E*) DNaseI of *rrnB* P1–RNAP complexes. Colored dots indicate the last downstream position protected by RNAP, and the corresponding gel traces to the *right* of the gel image indicate the overall extent of protection. (*B*) DNaseI footprints of RP_C (lane *6*), RP_I (lane *4*), and RP_{AC} (lane *3*). (Lane *5*) DksA shifts RP_I from a downstream endpoint of +12 to +1. The template strand was ^{32}P-labeled at the 3′ (upstream) end. (*C*) Representative $KMnO_4$ footprint of *rrnB* P1. Positions −10, −11, and −13 on the template strand are reactive in RP_{AC} (lanes *1*,*2*, green trace at *right* of gel image) but are predominately unreactive in RP_I ± DksA (lanes *3*–*6*, blue and red traces), and therefore RP_I is closed. (*D*) DksA does not affect the DNaseI footprint of RP_C (complex formed at 12°C in the absence of competitor). (Lanes *3*,*4*) Circle shows the downstream endpoint of protection in the RP_C and RP_C plus DksA footprints (see also red and gray traces at *right*). (*E*) DksA does not affect the DNaseI footprint of RP_{AC} (complex formed at 37°C with ATP and CTP). (Lanes *3*,*4*) Green dot shows the endpoint of downstream protection in RP_{AC} (see also red and green traces at *right*). In *B*, *D*, and *E*, arrows mark the transcription start site, +1, and positions +1, +12, and +20 are indicated on the traces for reference. The A + G ladders are sequence markers. RNAP was 40 nM, and DksA was 3.5 μM in *B*–*D*. RNAP was 30 nM, and DksA was 1.5 μM in *E*. The details are in the Materials and Methods and the Supplemental Material.

In contrast to the situation at most promoters, RP_O is very short-lived at rRNA promoters (Barker et al. 2001a). DNA sequences in the core rRNA promoter are responsible for the short-lived nature of this complex, including a suboptimal contact between σ region 1.2 and the discriminator, the region between the −10 hexamer and the transcription start site (Haugen et al. 2006). DksA and its cofactor ppGpp further shift the promoter–RNAP

equilibrium in the dissociation direction, thereby inhibiting rRNA transcription (Barker et al. 2001a; Paul et al. 2004; Haugen et al. 2008). At promoters that form long-lived complexes but bind RNAP very slowly, DksA/ppGpp can regulate transcription initiation positively (Paul et al. 2005). These increases result from a combination of direct and indirect effects of DksA/ppGpp on transcription (Zhou and Jin 1998; Barker et al. 2001a,b; Paul et al. 2005). Thus, the specific effect of DksA/ppGpp depends on the intrinsic kinetic characteristics of the promoter.

Some insights into the role of ppGpp were obtained from the identification of substitutions in the subunits of RNAP that suppressed growth defects of strains lacking *relA* and *spoT*, the genes responsible for ppGpp synthesis (Bartlett et al. 1998; Barker et al. 2001b; Trautinger and Lloyd 2002; Murphy and Cashel 2003; Szalewska-Palasz et al. 2007). Numerous substitutions were located in the proposed DNA path in transcription elongation complexes and destabilized RNAP–promoter complexes (Barker et al. 2001b; Trautinger and Lloyd 2002), consistent with the model that ppGpp functions in this manner.

To understand the mechanism of DksA action, we examined its effects on occupancy of complexes formed by RNAP at an rRNA promoter, *rrnB* P1. The results indicated that DksA hinders conformational changes in RNAP and DNA during the transition from RP_C to RP_I. Identification of amino acid substitutions in RNAP that mimicked or altered the effect of DksA allowed construction and initial confirmation of a working model for the action of DksA on transcription initiation.

Results

DksA reduces occupancy of an rrnB P1 transcription initiation intermediate

To understand the mechanism of rRNA transcription inhibition by DksA, we examined its effect on footprints of the *rrnB* P1 promoter complex under different solution conditions in vitro. Manipulation of solution conditions has been used previously to examine intermediates by footprinting (e.g., Spassky et al. 1985; Cowing et al. 1989).

We previously identified three *rrnB* P1 promoter complexes—RP_C, RP_I, and RP_{AC} (Fig. 1A; see below; Newlands et al. 1991; Bokal. et al. 1995; Bartlett et al. 1998). These complexes exhibit the same upstream endpoint in DNAseI footprints (approximately −60) but different extents of downstream protection from DNaseI (Fig. 1B), DNA strand opening (Figs. 1C; Supplemental Fig. S1), and resistance to displacement by competitors like heparin (for review, see Haugen et al. 2008).

The complex formed at 12°C is closed (as judged by the absence of DNA reactivity to $KMnO_4$) (Supplemental Fig. S1), and downstream protection extends to approximately +1 (Fig. 1B, lane 6, white dot), similar to RP_C at other promoters (Cowing et al. 1989; Schickor et al. 1990; Haugen et al. 2008). Because DNaseI requires access to at least 6 bp 3′ to the site of cleavage (Suck and Oefner 1986), the actual downstream boundary of RP_C likely extends only to approximately −6.

The complex formed at 37°C is also closed (Fig. 1C, cf. lanes 4,5 and lanes 2,3, RP_{AC}, which is open [see below]), but DNA protection from DNaseI extends to approximately +12 (RP_I) (Fig. 1B, lane 4, blue dot), indicating an actual downstream boundary of approximately +6. Most promoters form a stable RP_O under these conditions (Haugen et al. 2008), but at *rrnB* P1, RP_O is unstable and represents only a minor fraction of the population at equilibrium (Fig. 1C, lanes 4,5 vs. lanes 2,3). RP_I likely forms transiently at other promoters but is not observed in footprints because RP_O is the predominant complex.

A competitor-stable complex at *rrnB* P1 can be trapped at 37°C by addition of ATP and CTP, the first two nucleotides in the transcript (Gourse 1988; Newlands et al. 1991; Borukhov et al. 1993). This complex, RP_{AC}, is open as judged by its reactivity to $KMnO_4$ (Fig. 1C, lanes 2,3), and protection from DNaseI extends downstream to approimately +20 (Fig. 1B, lane 3, green dot), similar to the downstream endpoint observed in RP_O at other promoters (for review, see Haugen et al. 2008).

We examined the effect of DksA on complexes formed by *rrnB* P1. DksA shifted the downstream endpoint of DNaseI protection observed at 37°C in the absence of ATP and CTP from approximately +12 to approximately +1 (Fig. 1B, lane 5, red dot, cf. blue dot in lane 4, absence of DksA). DksA also reduced the minimal level of reactivity to $KMnO_4$ observed at 37°C (Fig. 1C, lanes 6,7; cf. lanes 4,5, absence of DksA). These results suggest that DksA destabilizes RP_I at *rrnB* P1 directly or indirectly by reducing promoter contacts with RNAP between approximately –6 and +6, changing the predominant complex occupied at 37°C to one similar or identical to RP_C. The correlation between effects of DksA on RP_I occupancy, promoter complex lifetime, and transcription is consistent with the model that DksA exerts its effects on *rrnB* P1 by shifting occupancy from RP_I to RP_C.

These results also suggested that DksA does not destabilize RP_C, since the complex with the +1 downstream endpoint accumulated at 37°C in the presence of DksA. Consistent with this interpretation, DksA did not affect the DNaseI footprint of the complex formed by *rrnB* P1 at 12°C (Fig. 1D, cf. normalized extent of downstream protection of lanes 3 and 4 in scan at right, indicated by white dot in gel image). If DksA had destabilized RP_C, a complete loss of protection by RNAP would have been observed, since the RNAP–promoter complex would have dissociated into its free components. DksA also had no effect on the footprints of RP_{AC} at *rrnB* P1 (Fig. 1E, cf. lanes 3 and 4, extent of downstream protection indicated by green dot) or at two promoters where RP_O is occupied at equilibrium, *argI* and *lacUV5* (Supplemental Fig. S2). A shift in equilibrium occupancy of these open complexes to earlier transcription initiation intermediates by DksA would have resulted in a loss in protection from DNaseI between approximately +1 and approximately +20.

We also examined effects of ppGpp on RP_I (data not shown). ppGpp alone had little or no effect on the RP_I footprint, consistent with its relatively small effect on transcription in vitro in the absence of DksA. ppGpp also exerted little or no additional effect on the footprint in

the presence of DksA, consistent with its lack of additional effects on transcription when DksA concentrations are high (Paul et al. 2004).

Selection and identification of ΔdksA suppressor mutations in rpoBC

In addition to inhibiting some promoters, DksA activates others (in conjunction with ppGpp), including some that direct transcription of genes for biosynthesis and transport of amino acids (Paul et al. 2005). We proposed previously that DksA might reduce the free energy of negatively and positively regulated promoter complexes similarly, but that transcriptional output might differ, depending on the intrinsic kinetic properties of individual promoters (i.e., affinity for RNAP, stability of RP_O, etc.) (Paul et al. 2005). Therefore, we reasoned that identification of mutations affecting either activation or inhibition by DksA might provide insight into the mechanisms of both processes.

We identified residues in RNAP important for DksA function by selecting for suppressor mutations that allowed strains lacking *dksA* to form colonies within 2 d at 30°C on a medium lacking amino acids. Spontaneous suppressors appeared at a frequency of $\sim 10^{-4}$ to 10^{-5}, possibly by bypassing the *dksA* requirement for amino acid biosynthesis or transport (see Discussion; Paul et al. 2005). Sixty-seven mutants (*ΔdksA* suppressors) were analyzed.

Like strains lacking *dksA*, strains without *relA* and *spoT* are unable to plate on minimal medium lacking amino acids. Spontaneous *ΔrelAΔspoT* suppressors map in *rpoB* and *rpoC* (Bartlett et al. 1998; Barker et al. 2001b; Trautinger and Lloyd 2002; Murphy and Cashel 2003; Szalewska-Palasz et al. 2007), and (at a much lower frequency) in *rpoD* (Hernandez and Cashel 1995). Therefore, we measured linkage to *rpoBC* as a first step in mapping the *ΔdksA* suppressor mutations. In each of the 67 suppressor strains, replacement of the *rpoBC* region with wild-type *rpoBC* by transduction with P1*vir* resulted in loss of suppression (loss of growth on medium lacking amino acids). Furthermore, P1 transduction of *ΔdksA* suppressors to a fresh *ΔdksA* strain resulted in growth on minimal medium in all 16 cases where this was tested (Supplemental Material; Supplemental Table S2). Taken together, these results strongly suggested that the *ΔdksA* suppressor mutations were in *rpoBC*.

The *rpoB* and *rpoC* genes were sequenced from 52 isolates (Table 1). After elimination of duplicates, 29 different mutations were identified, representing 24 single amino acid substitutions (two of which coded for different substitutions for the same amino acid), one double substitution, three deletions, and one insertion. Twenty mutations are in *rpoC*, eight are in *rpoB*, and the double contains a mutation in each gene. Figure 2 displays the positions of *ΔdksA* suppressors superimposed on a model of RP_O that was based on the X-ray structure of the *Thermus aquaticus* fork-junction complex (Murakami et al. 2002; Lawson et al. 2004). The positions of most of the *ΔdksA* suppressors (21 out of 28) are clustered in, or very close to, the RNAP switch regions (Table 1).

The switch regions serve as a hinge at the base of the RNAP clamp and form a network of salt linkages to the bridge helix (BH) (Cramer et al. 2001; Gnatt et al. 2001; Vassylyev et al. 2007a). The switches may undergo conformational changes and/or folding transitions in the course of binding to DNA and have been proposed to coordinate clamp opening/closing during transcription initiation and elongation (Cramer et al. 2001; Gnatt et al. 2001; Mukhopadhyay et al. 2008). Switch region residues directly contact template strand DNA in models of RP_O and in the TEC (Lawson et al. 2004; Vassylyev et al. 2007a; Belogurov et al. 2008), but because there is no structure of RP_I, the complex on which DksA acts (see above), it remains to be determined if promoter DNA has bent toward the active site sufficiently to contact the switch regions directly in this intermediate.

Of the seven positions not in, or directly adjacent to, the switch regions, two (β′ H450R and β′ K650T) are likely near or in the DksA-binding site, and two others (β′ Δ212–217 and β′ Δ1185–1216) likely affect DNA interactions downstream from the position of the transcription initiation bubble. The other three positions are in the β subunit (Y395D, R454H/R454L, and H551P), and their selection might also be rationalized by effects on the DNA path (e.g., β H551P is in fork loop 2, between the separated strands of DNA and adjacent to the BH in RP_O) (Fig. 2; Lawson et al. 2004).

Eighteen of the suppressors are near the template strand in the model of RP_O between positions approximately −2 and approximately +6, and three are near the nontemplate strand between approximately −2 and approximately +6, correlating with the region of DNA differentially protected in the footprints of RP_C versus RP_I.

In contrast to the *ΔdksA* suppressors, a large majority of which are in β′, a majority of the *ΔrelAΔspoT* suppressors are in β. Furthermore, 22 of the 29 *ΔdksA* suppressors were not identified previously as suppressors of *ΔrelAΔspoT* strains (Supplemental Table S3; Bartlett et al. 1998; Trautinger and Lloyd 2002; Murphy and Cashel 2003; Szalewska-Palasz et al. 2007). As noted previously (Trautinger and Lloyd 2002), many of the *ΔrelAΔspoT* suppressors are spread along the main channel of RNAP. Those that have been tested reduce the lifetimes of promoter complexes (Bartlett et al. 1998; Zhou and Jin 1998; Barker et al. 2001b; Trautinger et al. 2005; Szalewska-Palasz et al. 2007), supporting the model that ppGpp functions by reducing promoter complex lifetime (see Discussion for further comparison of the *ΔdksA* and *ΔrelAΔspoT* suppressors).

In summary, we propose that switch region residues play a central role in DksA function, accounting for their identification in our selection. Furthermore, because RP_I is the complex on which DksA funcitons, these data suggest that the switch regions might affect the RP_C-to-RP_I transition by controlling clamp opening to allow DNA entry and/or by establishing contacts directly with promoter DNA at this step in the transcription initiation mechanism.

ΔdksA suppressors reduce rrnB P1 transcription

To test whether the *rpoBC* mutants mimic the effects of *dksA* in vivo and thus compensate for its absence, we measured effects of five *ΔdksA* suppressors (including representatives in RNAP switch regions 1 [β′ F1325L], 2

Table 1. *Substitutions in RNAP allowing growth of* ΔdksA *cells on minimal medium lacking amino acids*

Subunit[a]	Substitution[b]	Isolates[c]	RNAP region[d]	Previously reported[e]
Single amino acid substitutions				
β	Y395D	1	Lobe	f
β	R454H	7	Protrusion (lobe/fork)	g,h
β	R454L	2	Protrusion (lobe/fork)	g
β	H551P	1	Fork loop 2	f,g
β	Q1264P	1	Switch 3	g
β	G1267V	1	Switch 3	
β	R1269H	1	Switch 3	
β	P1317L	5	Near ω, 4 Å from switch 4	
β′	G333D	1	Switch 2	
β′	K334I	1	Switch 2	
β′	G336C	1	Switch 2	
β′	R337S	5	Switch 2	
β′	L343I	1	Switch 2	
β′	D348Y	1	Switch 2	g
β′	H450R	1	Active site region	
β′	K650T	1	Secondary channel rim	
β′	R799L	1	Bridge helix, touching switch 1	
β′	Q805P	1	Bridge helix, 5 Å from switch 1	
β′	G1308C	1	Switch 1	
β′	L1314Q	1	Switch 1	
β′	S1324L	3	Switch 1	
β′	F1325L	3	Switch 1	
β′	F1325V	1	Switch 1	
β′	G1354C	1	Near ω, 6 Å from switches 4, 5	
Double amino acid substitutions				
β/β′	R1246C/K334Q	1	Switch 3/switch2	
Insertions and deletions				
β′	Δ212–217	1	Inside clamp head	h, i
β′	Ω320–324[j]	1	Rudder, 7 Å from switch 2	
β′	Δ1185–1216	2	Cleft region	
β′	Δ1335–1337	1	Inside clamp core, touching switch 1	

[a]*rpoB* encodes β; *rpoC* encodes β′.
[b]Amino acid substitution (original amino acid followed by the residue number and the new amino acid).
[c]Number of isolates.
[d]Location of substitution (as defined by structural alignment with yeast RNA pol II (Cramer et al. 2001).
[e]Previously isolated as suppressor of amino acid auxotrophy of Δ*relA*Δ*spoT* mutant.
[f]Trautinger and Lloyd 2002.
[g]Szalewska-Palasz et al. 2007.
[h]Bartlett et al. 1998.
[i]Murphy and Cashel 2003.
[j]Tandem duplication of β′ residues 320–324.

[β′ R337S], and 3 [β G1267V; β R1269H], and the BH [β′ Q805P]) on expression of *rrnB* P1-*lacZ* fusions. Consistent with our previous observations (Paul et al. 2004), deletion of *dksA* in a strain wild-type for *rpoBC* increased *rrnB* P1 activity approximately fourfold in log phase (Fig. 3A). In contrast, *rrnB* P1 activity in *ΔdksA* strains containing the suppressor mutations was similar to that in the strain with wild-type *dksA* and wild-type *rpoBC* (Fig. 3A). Thus, the *rpoBC* mutations mimicked the effect of wild-type *dksA* on *rrnB* P1 in vivo, without inhibiting the activities of two control promoters, *lacUV5* and *argI* (Supplemental Fig. S3).

To determine if the effects of the suppressor mutations on rRNA promoter activity were direct, we measured transcription in vitro by eight of the mutant RNAPs (including the five tested in vivo). Seven of the purified RNAPs contained substitutions in or near the switch regions or BH, and one contained a substitution near the position of ω (which has been implicated in ppGpp function) (Vrentas et al. 2005). In each case, transcription from *rrnB* P1 was much lower than transcription by wild-type RNAP, whereas transcription from a control promoter, *RNA1*, was relatively unaffected by the RNAP substitutions. Transcription from *rrnB* P1 by the mutant RNAPs was even lower than by wild-type RNAP in the presence of DksA and ppGpp (Fig. 3B). Thus, effects of the *rpoBC* mutations on *rrnB* P1 are promoter-specific and direct.

ΔdksA suppressors shift the $RP_C \leftrightarrow RP_I$ equilibrium in the dissociation direction

DksA reduces the lifetimes of complexes formed at all promoters that have been examined, shifting the equilibrium

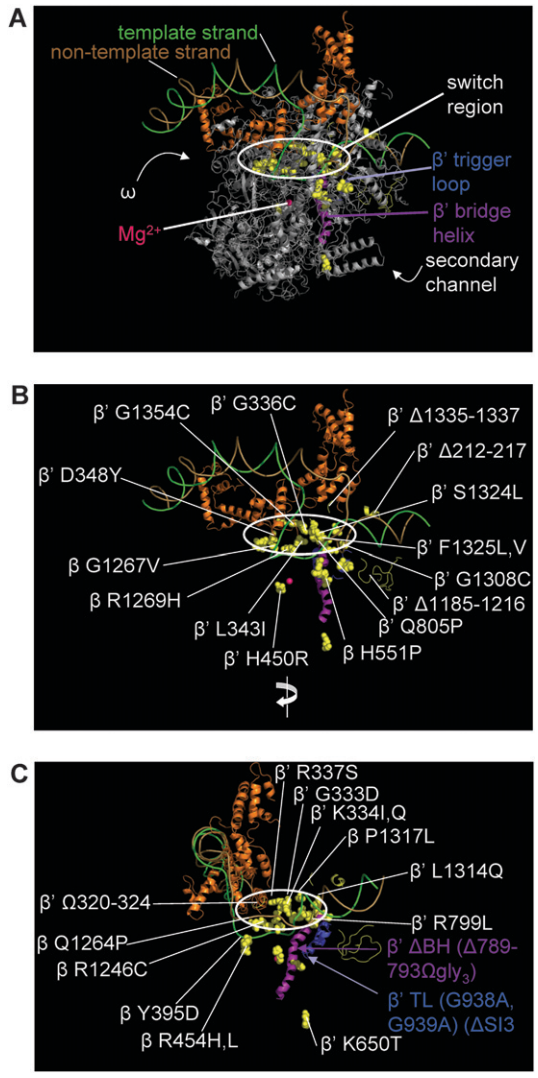


Figure 2. Substitutions that suppress the effects of the *ΔdksA* mutation cluster in the switch and BH regions of RNAP near the transcription start site. (*A*) Model of RP_O based on the *T. aquaticus* fork-junction complex (Murakami et al. 2002; Lawson et al. 2004). In this view, upstream DNA enters the enzyme at the top left, and downstream DNA exits the main channel to the right. β, β′, ω, and α_2 are gray; σ is orange; template strand is green; nontemplate strand is brown; active site Mg^{2+} (Mg1) is a magenta sphere; β′ BH is purple; and β′ TL is light blue (TL is more visible in *C*). Arrows point to ω (on back face) and the secondary channel (underside). Positions of substitutions that allow *ΔdksA* mutants to grow in the absence of amino acids are in yellow spacefill. Positions of deletions are also indicated in yellow. The oval shows the approximate boundary of the switch regions. In *B*, the orientation is the same as in *A*, but β, β′, ω, and α_2 have been omitted, except for the residues in yellow (*ΔdksA* suppressors), the BH, and TL. In *C*, RNAP is rotated 90°. Deletions and insertions are indicated by Δ and Ω, respectively. Approximate positions of site-directed mutants in the BH and TL, discussed later in the text, are also indicated.

in the dissociation direction and inhibiting transcription from promoters that form intrinsically short-lived complexes (Paul et al. 2004; Rutherford et al. 2007; Haugen et al. 2008). To determine whether the *ΔdksA* suppressors in *rpoBC* altered the lifetimes of promoter complexes, we tested the effects of seven mutant RNAPs on complexes formed by the *lacUV5* and *argI* promoters (Barker et al. 2001b; Paul et al. 2005). (The decay rates of *rrnB* P1 complexes with the mutant RNAPs were too fast for us to measure them accurately [data not shown].) The mutant RNAPs formed complexes with the *lacUV5* and *argI* promoters that were as much as 50-fold shorter-lived than the complexes formed by wild-type RNAP (Fig. 3C,D and legend). Complexes made by the mutant RNAPs in the absence of DksA and ppGpp had lifetimes similar to those made by wild-type RNAP in the presence of DksA and ppGpp.

We also performed DNaseI footprints on *rrnB* P1 with four mutant RNAPs in the absence of DksA to determine whether the protection pattern was the same as with wild-type RNAP in the presence of DksA. Under conditions in which wild-type RNAP without DksA formed a complex that extended to approximately +12 (RP_I), and wild-type RNAP with DksA made a complex that extended to approximately +1 (Fig. 3E, lanes 4,5), the mutant RNAPs (β′ G333D) (Fig. 3E, lane 6; β′ R337S, β′ F1325V, and β′ G1354C; data not shown) made complexes that extended only to approximately +1. We conclude from both the lifetime and footprint assays that the suppressor substitutions mimic the effect of DksA on wild-type RNAP by altering interactions between RNAP and the promoter, shifting the $RP_C \leftrightarrow RP_I$ equilibrium toward dissociation, and that the switch regions and BH play an essential role in this step in transcription initiation.

Role of the trigger loop (TL) in DksA signal transmission

We propose that DksA ultimately targets interactions between RNAP and promoter DNA either by targeting residues that affect the clamp conformation during RP_I formation or that directly contact DNA in RP_I. In either case, these RNAP residues in the switch regions are 25–40 Å away from where DksA binds in the secondary channel. Thus, signals must be propagated allosterically. Since sections of both the TL and the ~60-Å-long BH likely are accessible to DksA in the secondary channel (Vassylyev et al. 2002, 2007a), they are candidates for transmitting the conformational changes.

The TL is located at the bottom of the secondary channel, assumes multiple conformations during nucleotide addition (Vassylyev et al. 2007b), and is positioned to interact with the BH (see below). To address the role of the TL in DksA function, we examined effects of DksA on two previously constructed mutant RNAPs (Toulokhonov et al. 2007). In both constructs, β′ contains an alteration in the TL and lacks sequence insertion 3 (SI3; residues 943–1130), a region of β′ that interrupts the TL in *Escherichia coli* but not in *Thermus* RNAP (Vassylyev et al. 2007b). In one mutant, β′ΔTL(ΔSI3), the TL and SI3 (β′ residues 931–1137) are replaced by three alanine residues. In the other, β′TL GG → AA(ΔSI3), SI3 is deleted, and the TL contains two alanine substitutions (G938A and G939A) that result in an altered TL conformation

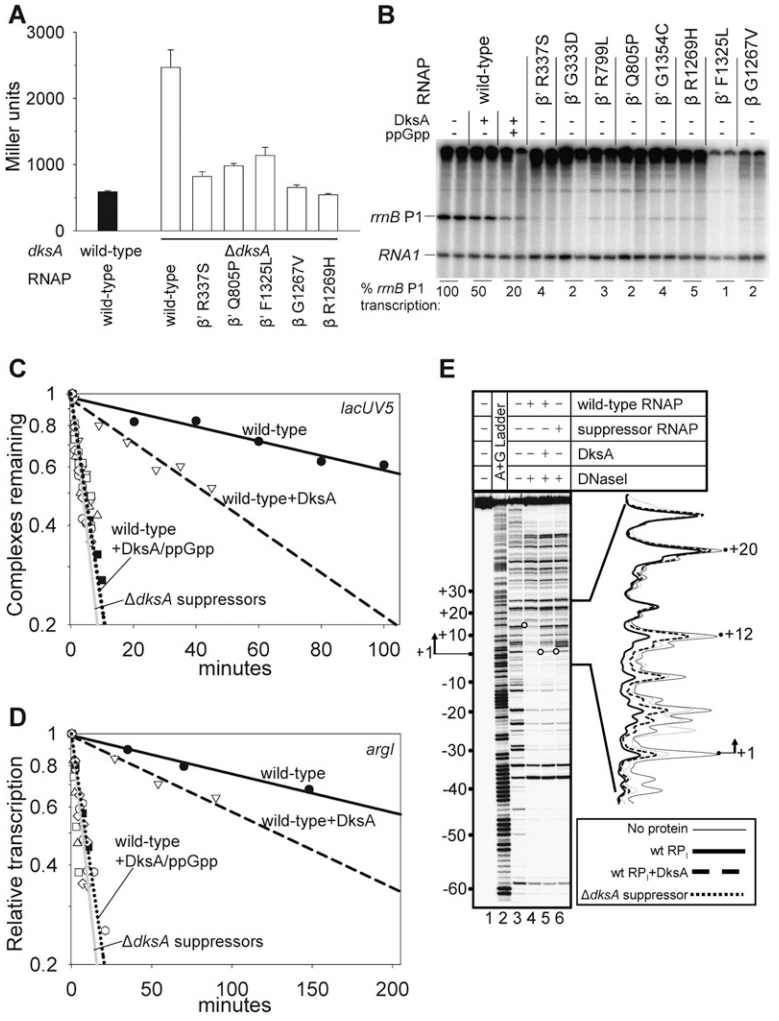


Figure 3. *ΔdksA* suppressors mimic the effect of DksA on *rrnB* P1 transcription. (*A*) *rrnB* P1 promoter activities in Δ*dksA* strains containing wild-type or mutant *rpoB*/*rpoC* alleles. β-Galactosidase activities from *rrnB* P1-*lacZ* fusions (three or more replicate cultures). (*B*) Transcription from *rrnB* P1 by *ΔdksA* suppressor RNAPs in vitro. Relative transcription (wild-type RNAP = 100%) is indicated ±ppGpp/DksA (average of duplicate lanes; see the Materials and Methods and the Supplemental Material). (*C*,*D*) Lifetimes of promoter complexes formed by Δ*dksA* suppressor RNAPs. The half-lives in *C* were measured using a filter binding assay and in *D* using in vitro transcription as a readout, as described in the Materials and Methods and the Supplemental Material. Plots show the fraction of complexes remaining (or transcription) as a function of time after heparin addition for the *lacUV5* (*C*) and *argI* (*D*) promoter. Absolute half-life (min) and error values in *C*, the *lacUV5* complex, were wild-type RNAP, 106 ± 20; wild-type RNAP with DksA, 38 ± 7.1; wild-type RNAP with DksA and ppGpp, 5.6 ± 1.2. For clarity, an average regression line is provided for the eight *ΔdksA* suppressor mutant RNAPs listed in *B*; the absolute half-life values ranged from 3.4 ± 0.3 to 6.2 ± 0.8. Absolute half-lives for *D*, the *argI* complex, are in Figure 5C. (*E*) DNaseI footprints at 37°C of a representative *ΔdksA* suppressor RNAP, β′ G333D, on *rrnB* P1. The conditions are indicated above each lane and in the Materials and Methods. Open circles indicate the extent of downstream protection with wild-type RNAP (lane *4*, thick line in scan at *right*), with wild-type RNAP + DksA (lane *5*, dashed line in scan), and with mutant RNAP (lane *6*, dotted line in scan). Note the absence of protection in the region from +1 to approximately +12 with the mutant RNAP. Footprints with the β′ R337S, β′ F1325L, and β′ G1354C RNAPs were similar to that with β′ G333D RNAP. DksA was 2 μM, and ppGpp was 100 μM.

and a dramatic decrease in the rate of NTP addition during transcription elongation (Vassylyev et al. 2007b).

We compared the lifetimes of promoter complexes formed by β′ΔTL(ΔSI3), β′TL GG → AA(ΔSI3), β′ ΔSI3, or wild-type RNAP in the absence and presence of DksA (Fig. 4). Whereas DksA decreased the lifetimes of the wild-type, β′ΔSI3, and β′TL GG → AA(ΔSI3) RNAPs fivefold to ninefold (Fig. 4A–C), DksA had little or no effect on the β′ΔTL(ΔSI3) RNAP (Fig. 4D), suggesting that the TL is needed for DksA to bind RNAP and/or perform a subsequent step in DksA function. The slightly shorter-lived complex that the β′ΔTL(ΔSI3) RNAP forms with the promoter (approximately threefold compared with the β′ΔSI3 RNAP, which is DksA-responsive) is insufficient to account for the absence of an effect of DksA, since RNAP mutants that decrease the relative lifetime of promoter complexes far more than the β′ΔTL(ΔSI3) RNAP still respond to DksA (Fig. 5C; data not shown). To address whether DksA binds to β′ΔTL(ΔSI3) RNAP, we used an assay in which hydroxyl radicals are generated at the RNAP active center by replacement of Mg^{2+} with Fe^{2+}, which results in cleavage of the coiled-coil tip of DksA (Perederina et al. 2004). DksA was cleaved similarly by the β′ΔTL(ΔSI3) RNAP and the wild-type RNAP, indicating it bound to both RNAPs (Fig. 4E). These results suggest that an interaction of DksA with the TL may be required to generate the allosteric signal transmitted from the secondary channel to RNAP residues that interact with DNA.

The complexes formed by the β′TL GG → AA(ΔSI3) and β′ΔSI3 RNAPs were threefold to fourfold shorter-lived than the wild-type RNAP complexes (Fig. 4A–C). To determine whether these mutant RNAPs were more sensitive to DksA than the wild-type RNAP, the DksA concentration dependencies of the promoter complex lifetimes were compared. β′TL GG → AA(ΔSI3) RNAP required ~0.1 μM DksA for half-maximal reduction of complex lifetime, whereas β′ΔSI3 RNAP and wild-type RNAP required ~0.3–0.4 μM DksA (Fig. 4F). These results suggest that the β′ G938A and G939A TL substitutions (and not the SI3 deletion) favor a TL conformation that facilitates transmission of the DksA-binding signal to its site of action.

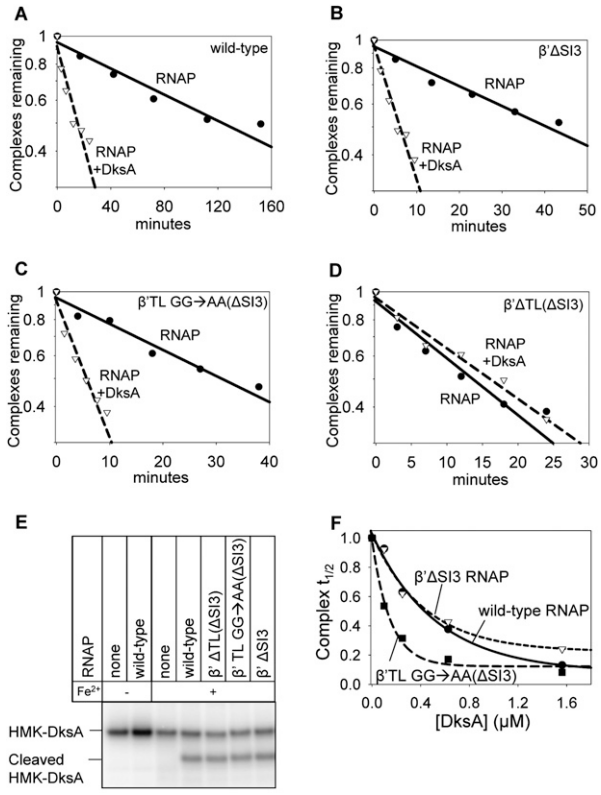


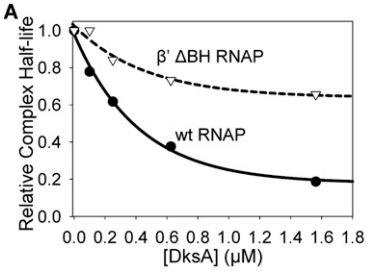
Figure 4. Effects of TL mutants on DksA function. (*A–D*) Half-lives of RNAP complexes containing the *lacUV5* promoter were measured by filter retention. Plots show the fraction of complexes remaining as a function of time after heparin addition. The fold-decrease in the half-life of the complex resulting from addition of 1.6 μM DksA is in parentheses: (*A*) wild-type RNAP (8.1 ± 2.4), (*B*) β′ ΔSI3 RNAP (5.3 ± 1.3), β′ TL GG → AA(ΔSI3) RNAP (8.6 ± 3.7), β′ΔTL(ΔSI3) RNAP (0.93 ± 0.04). (*E*) Fe^{2+} cleavage assay for DksA binding to wild-type and mutant RNAPs. (*F*) The concentration dependence of effects of DksA on the lifetimes of *lacUV5* promoter complexes were measured with wild-type or mutant RNAPs. See the Supplemental Material for further information.

Role of the BH in DksA signal transmission

The BH is accessible through the secondary channel, and it interacts with the TL. Furthermore, the C-terminal region of the BH extends to the switch region and may contact promoter DNA near the transcription start site (Gnatt et al. 2001). Thus, the BH is a candidate for transmitting the signal initiated by DksA binding in the secondary channel. To address the role of the BH, we tested effects of DksA on a mutant RNAP in which five amino acids near the center of the BH at the base of the secondary channel were replaced by a glycine linker (β′ Δ789–793Ωgly3, referred to as β′ ΔBH) (see Fig. 2C; Toulokhonov et al. 2007). Even at the highest concentrations of DksA used, where effects on promoter complex lifetime were saturating for wild-type RNAP, DksA had much smaller effects on the β′ΔBH RNAP than on wild-type RNAP (Fig. 5A). The dramatic effect of the ΔBH mutation on the effect of DksA could not be explained by an absence of DksA binding, since Fe^{2+}-mediated cleavage of DksA was still observed with the mutant enzyme (Fig. 5B). We conclude that the BH contributes to the network of interactions that transmits effects of DksA binding to the promoter.

A switch mutant resistant to the effect of DksA

Effects of the *ΔdksA* suppressors on the RP_C-to-RP_I transition suggested that the switch region substitutions might perform the same function as DksA, either affecting clamp opening/closing or reducing direct RNAP–promoter contacts in RP_I. Furthermore, our data suggested that effects of DksA binding in the secondary channel might be transmitted to the switch regions via the TL and the BH. We next addressed whether any of the positions in the switches identified by the *ΔdksA*



C

RNAP	t1/2 (min)	t1/2 (+/-DksA)
wt	239±22	0.54±0.14
β′ G333D	5.98±1.1	0.57±0.13
β′ R337S	22.0±10	0.53±0.18
β′ R799L	8.5±2.1	0.49±0.20
β′ Q805P	4.2±0.8	0.49±0.05
β′ F1325L	8.0±1.2	0.41±0.07
β′ G1354C	11.6±3.0	0.38±0.09
β G1267V	8.5±3.2	1.00±0.24

Figure 5. Mutant RNAPs defective in the response to DksA. (*A*) Substitutions in the BH decrease the effect of DksA on promoter complex lifetime. The concentration dependence of effects of DksA on the lifetimes of *lacUV5* promoter complexes were measured with wild-type or ΔBH RNAP. See the Supplemental Material for further information. (*B*) DksA binding was measured by Fe^{2+}- mediated cleavage (see the Materials and Methods and the Supplemental Material) for wild-type, β′ ΔBH, and β G1267V RNAPs. (*C*) Half-lives of complexes containing wild-type or mutant RNAPs on the *argI* promoter (see the Materials and Methods). For each RNAP, the value in column 2 is the absolute half-life, and the value in column 3 is the ratio of the half-life ±2.25 μM DksA.

suppressor mutations were resistant to DksA function and therefore, like the TL and BH, might be on the signal transduction pathway. We tested the effects of DksA on the complexes formed by seven *ΔdksA* suppressor RNAPs. Each of the mutant RNAPs made promoter complexes with short half-lives. However, DksA further reduced the lifetimes of the complexes formed by six of the seven mutants, indicating that these residues are not essential for DksA function (Fig. 5C).

β G1267V RNAP (a substitution in switch region 3) was unaffected by DksA (Fig. 5C). DksA was cleaved by Fe^{2+} in the active site of the mutant RNAP (Fig. 5B), suggesting that the defect occurred subsequent to DksA binding. Although further studies will be needed to rule out quantitative differences in binding at low DksA concentrations, we conclude that β G1267 might play a direct role in the network of promoter–RNAP interactions targeted by DksA (see Discussion).

Discussion

Promoter–RNAP interactions in RP_C and RP_I

The unusual kinetic properties of *rrnB* P1 made it possible to study effects of DksA on occupancy of three different complexes: two closed complexes, RP_C (highly occupied at 12°C) and RP_I (highly occupied at 37°C); and an open complex, RP_{AC} (stabilized by the addition of the first two NTPs in the transcript). As with the closed complex formed at low temperatures at other promoters (e.g., Cowing et al. 1989; Schickor et al. 1990), RP_C is characterized by a shortened DNaseI downstream footprint (to +1), relative to that in RP_{AC} at *rrnB* P1 (to +20) and in RP_O at other promoters. This indicates that promoter DNA in RP_C has not undergone the sharp bend required for entrance into the active site cleft of the enzyme (Murakami et al. 2002; Saecker et al. 2002; Murakami and Darst 2003).

In contrast, DNaseI protection extends to approximately +12 in RP_I, indicating an actual downstream boundary of the complex of approximately +6, but the strands remain closed. The DNA in the complex displays uniform protection in hydroxyl radical footprints from the −10 hexamer to at least +6 (Supplemental Fig. S4), not the periodic protection observed upstream of the −10 hexamer that is characteristic of binding of RNAP to a single face of the double helix. The hydroxyl radical footprint protection pattern suggests that the DNA duplex in and downstream from the transcription start site is enclosed within the enzyme. Although *rrnB* P1 RP_I resembles some other previously reported closed intermediates—e.g., λP_R I_1 (Saecker et al. 2002) and *lacUV5* RP_I (Spassky et al. 1985)—the extent of downstream DNaseI protection in those intermediates was reported to extend further downstream (to at least +20).

The DNA duplex in a model of λP_R I_1, an intermediate in RP_O formation, has an ~90° bend in the −10 region. In this model, DNA downstream from approximately −5 is enclosed within the enzyme but is positioned ~50 Å above the active site (Saecker et al. 2002). The precise path of duplex DNA in λP_R I_1 (or in *rrnB* P1 RP_I) is not known, but interactions with RNAP in λP_R I_1 were proposed to result in conformational changes leading to a second unstable intermediate, I_2. Additional conformational changes were proposed to result in formation of a stable RP_O (Kontur et al. 2008). RP_I may be an intermediate immediately preceding I_1 in which the clamp has opened to accommodate entry of dsDNA.

We identified multiple substitutions in the RNAP switch regions and BH that inhibited the transition from RP_C to RP_I at *rrnB* P1, and thereby mimicked the effects of DksA (Table 1; Figs. 2, 3). In the TEC, most template strand interactions between RNAP and the transcription bubble from approximately −6 to approximately +4 (with respect to the position of the active site) are mediated by residues in the switch regions and BH, whereas duplex DNA downstream from the transcription bubble interacts with the mobile clamp (Gnatt et al. 2001; Vassylyev et al. 2007a). We emphasize that because we do not know the exact path of the DNA in RP_I, we do not know whether the residues identified in our *ΔdksA* suppressor selection directly affect interactions with DNA in RP_I or indirectly affect interactions with DNA in RP_I by controlling opening/closing of the clamp domain.

An allosteric model for DksA function

No structure is available for DksA bound to RNAP, and there is no apparent DksA homolog in *Thermus thermophilus* or *T. aquaticus* (Perederina et al. 2004), the only species from which high-resolution bacterial RNAP structures have been solved. However, there is substantial evidence that the coiled-coil tip of DksA is located near the active site and TL in the *E. coli* RNAP secondary channel: (1) DksA is cleaved when Mg^{2+} in the active site is replaced by Fe^{2+} (Figs. 4, 5; Perederina et al. 2004); (2) DksA protects regions of RNAP in the secondary channel near the active site in protein–protein footprints (I. Toulokhonov and R.L. Gourse, unpubl.); and (3) GreB can function like DksA in rRNA regulation when provided at sufficiently high concentrations (Rutherford et al. 2007).

The apparent location of DksA in the RNAP secondary channel and the position of the RNAP–promoter interactions affected by DksA downstream from the −10 region in RP_I imply that the effects of DksA are allosteric. Effects of substitutions in the TL and the BH on DksA function suggest that DksA bound in the secondary channel might interact directly with one or both of these structural elements and cause conformational changes that destabilize RNAP–DNA interactions at least 25–40 Å away (Fig. 6). Possible targets of the conformational changes that are initiated by DksA binding in the secondary channel were suggested by the positions of RNAP substitutions that mimicked the effects of DksA on RP_I, including residues in the switch regions and BH. β G1267V not only mimicked effects of DksA but also eliminated further effects of DksA, suggesting that it might be at/near the end of the pathway for transmission of the effects of DksA to the promoter (Fig. 5). Positions in the switch regions could affect promoter interactions with RNAP through direct interactions with DNA, by

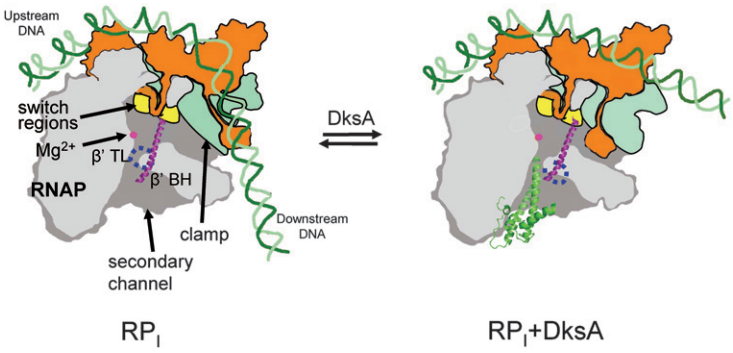


Figure 6. Model for DksA action on *rrnB* P1 RP_I. Switch regions (yellow), β′ TL (blue), β′ BH (purple), downstream clamp (light green), active site Mg^{2+}(magenta sphere), remainder of RNAP (gray), DksA (dark green). DNA strands are dark green (template) and light green (nontemplate). Schematic model modified by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Haugen et al. 2008) (© 2008). (*Left*) DNA bends toward the active site in the absence of DksA to form RP_I (downstream boundary of approximately +6), with the TL (dotted blue circle) oscillating between multiple conformations. (*Right*) DksA affects conformation of the TL and BH, ultimately affecting the switch regions. Changes in the switches alter clamp conformation and thereby indirectly affect DNA interactions and/or directly affect DNA contacts. This leads to decreased occupancy of RP_I and increased occupancy of RP_C (downstream boundary of approximately −6).

interacting with DNA-binding residues, or by controlling opening/closing of the clamp domain. Understanding the details of the mechanism will depend on the precise interactions between DksA and RNAP, the trajectory of DNA, and the positioning of the RNAP clamp, which are not yet known in RP_I.

We found that the TL is needed for DksA function, consistent with the prediction that the proximity of the TL to the secondary channel could result in regulation of transcription by factors that bind there (Vassylyev et al. 2007b). Because the substitutions in the β′TL GG → AA (ΔSI3) RNAP favor an altered TL conformation (Vassylyev et al. 2007b) and we observed that these substitutions enhanced DksA function (Fig. 4), we suggest that this conformation increases DksA binding and/or more efficiently transmits the effects of DksA binding to the switch regions of RNAP. Effects of DksA also appear to be mediated by the BH (Fig. 5). Thus, the overall picture that emerges is that DksA interacts with certain conformational states of the TL and/or BH that inhibit RP_I formation. These TL and/or BH conformations could be a subset of those that occur in core RNAP and that play roles in the NTP addition cycle (Vassylyev et al. 2007b). Interactions of DksA with the TL and/or BH probably form only part of a larger network of interactions contributing to the mechanism of DksA action.

Recently, it was found that the antibiotics myxopyronin, corallopyronin, and ripostatin bind to the RNAP switch regions and inhibit transcription initiation (Belogurov et al. 2008; Mukhopadhyay et al. 2008). Although there was agreement that the antibiotics bound to the switch regions, there was less agreement concerning the mechanism of transcription inhibition. The two models proposed for inhibition by the antibiotics (i.e., antibiotic–switch interactions prevent opening of the clamp to allow DNA entry versus antibiotic–switch interactions directly interfere with promoter DNA interactions) are similar to the two potential mechanisms suggested above for the effects of DksA. However, we propose that DksA targets the switch regions allosterically rather than by binding to them directly.

The proximity of the BH to both the switch regions and the secondary channel and the identification of BH mutants resistant to DksA support the interpretation that the BH links effects of DksA in the secondary channel to the switch regions. However, we cannot rule out the possibility that the *ΔdksA* suppressor mutations bypass the need for DksA by a completely independent mechanism; i.e., that DksA does not actually affect RP_I stability by altering the conformation of the switches/BH, even though the end result is the same.

We suggest that the ability of DksA to decrease the lifetimes of complexes formed by all promoters is ascribable to its effects on the RP_C-to-RP_I transition. At promoters that form stable RP_O complexes, DksA pulls the $RP_I \leftrightarrow RP_O$ equilibrium in the dissociation direction, although this does not inhibit transcription initiation because RNAP escapes from the promoter before a major fraction of RP_O has decayed (Paul et al. 2004; Haugen et al. 2006).

It was proposed previously that DksA might bind to an intermediate between RP_C and RP_O and prevent folding of the clamp around downstream promoter DNA (Kontur et al. 2006), a model consistent with the shorter RP_I footprint that we observed in the presence of DksA. However, our analysis indicates that DksA decreases the lifetime of RP_I, thereby shifting RP_I back to RP_C, decreasing RP_O indirectly by reducing occupancy of RP_I, consistent with the absence of effects of DksA on footprints of RP_O (Supplemental Fig. S2).

Role of ppGpp in DksA function

Changes in the concentrations of ppGpp and NTPs result in rRNA promoter regulation under different nutritional conditions (Murray et al. 2003). However, modification of RNAP by DksA is required for these small molecules to exert their effects on transcription initiation (Paul et al. 2004, 2005), accounting for large effects of *dksA* on regulation in vivo even though its concentration is relatively constant (Rutherford et al. 2007). When DksA concentrations are not saturating, we showed previously that DksA and ppGpp function synergistically (Paul et al. 2004, 2005). We proposed that together they lower the free energy of an intermediate and/or transition state on the pathway to RP_O formation, and that differences in the intrinsic kinetics of individual promoters determine

whether transcriptional output decreases, increases, or stays the same. However, models for the mechanism of ppGpp action and how ppGpp synergizes with DksA await identification of the biologically significant ppGpp-binding site on RNAP (Vrentas et al. 2008).

Mechanism of ΔdksA suppression

The basis for the *ΔdksA* auxotrophy (and the suppression of this auxotrophy) could be quite complex. Many cellular promoters require *dksA* for regulation (for review, see Magnusson et al. 2007; Haugen et al. 2008), among which are several for amino acid synthesis or transport (Paul et al. 2005). DksA and ppGpp also directly or indirectly affect DNA replication/repair (Trautinger and Lloyd 2002; Trautinger et al. 2005). Consistent with the ability of the *ΔdksA* suppressors to compensate for the absence of amino acids in the growth medium, they increased the activity of at least one amino acid biosynthetic promoter in vivo (Supplemental Fig. S3). However, a subset of the suppressors also increased the activity of the *lacUV5* promoter, whose activity with wild-type RNAP is not increased by DksA/ppGpp. Therefore, further studies will be needed to determine if the mutant RNAPs bypass the loss of *dksA* by stimulating amino acid promoter activity.

As indicated above (see also Supplemental Table S3), a relatively small number of the *ΔdksA* suppressors were also isolated as *ΔrelAΔspoT* suppressors in previous selections (e.g., Bartlett et al. 1998; Trautinger and Lloyd 2002; Murphy and Cashel 2003; Szalewska-Palasz et al. 2007). As a further test of the qualitative overlap in the two pools of mutants, we analyzed five mutants isolated only as *ΔdksA* suppressors (indicated with asterisks in Supplemental Table S3) and two additional *ΔrelAΔspoT* suppressors (in *rpoD*) (Hernandez and Cashel 1995) for their abilities to suppress the nonselected phenotype (see "Expanded Experimental Procedures" in the Supplemental Material). All five *ΔdksA* suppressors suppressed the amino acid auxotrophy of the *ΔrelAΔspoT* mutant, and the two additional *ΔrelAΔspoT* suppressors weakly suppressed the *ΔdksA* mutant (Supplemental Table S3). Therefore, it is possible that all the mutants would (at least weakly) suppress the auxotrophy of the strain in which it was not originally selected.

The absence of complete overlap in the *ΔrelAΔspoT* and *ΔdksA* suppressor sets, and yet their suppression of the other mutant strain when tested, could be explained by the fact that neither selection was saturated: The different suppressor sets could represent different subsets of the same mutant population. However, the different suppressor sets (and their likely differential suppression in quantitative terms of the host strains used for their selection) probably reflect differences in the requirements for DksA and ppGpp for expression of specific target genes (Murray et al. 2003; Paul et al. 2004; Magnusson et al. 2007) or for maintaining genome integrity (Trautinger et al. 2005). In this regard, we note that different selections or screens were used in some cases for mutant identification (Trautinger and Lloyd 2002; Szalewska-Palasz et al. 2007).

Future prospects

We have presented a model for the mechanism of DksA function in transcription initiation. Although our model can explain the basic elements of negative regulation, we still lack a structural framework for understanding how the same conformational changes could lead to positive control of transcription initiation and for understanding the mechanism of DksA synergy with ppGpp. Additional studies will be needed to expand on the basic framework provided here.

Materials and methods

Strains, plasmids, and primers

Strains, plasmids, and primers are listed in Supplemental Tables S1 and S4.

RNAP and DksA purification

Wild-type and mutant RNAPs were purified as described in the Supplemental Material. The methods produced enzymes that responded to DksA and ppGpp identically (Vrentas et al. 2005). His_6-tagged DksA was purified as described (Paul et al. 2004).

DNaseI and $KMnO_4$ footprinting

DNaseI and $KMnO_4$ footprints were performed essentially as described (Newlands et al. 1991; Bartlett et al. 1998). PhosphorImager traces were obtained by quantifying each lane, normalizing to a region of the DNA not affected by RNAP or DksA. Footprints were performed multiple times, and representative gels are shown. See the Supplemental Material for details.

Selection, mapping, and identification of ΔdksA suppressor mutations

See "Expanded Experimental Procedures" in the Supplemental Material for details. Briefly, a *ΔdksA* strain was plated on a medium lacking amino acids at 30°C. Colonies (*ΔdksA* suppressors) appeared after ~2 d at a frequency of $\sim 10^{-5}$ or 10^{-6}. All 67 suppressor mutations tested were linked to *rpoBC*. The *rpoBC* region was amplified from 52 suppressor strains by PCR of 10 overlapping portions of each gene, using the primers listed in Supplemental Table S4, and sequenced with the same primers. Mutations were identified in 49 strains (Table 1; the strain numbers are in Supplemental Table S2).

Promoter activity assays

Promoter activities were determined in vivo by measuring β-galactosidase activities produced from *rrnB* P1(endpoints −61, +1)-*lacZ* fusions (Barker et al. 2001a) from cells grown at 30°C in MOPS medium with 0.4% glycerol and 40 μg/mL each of tryptophan and tyrosine and 80 μg/mL each of the other 18 amino acids. Promoter activities were determined in vitro from multiple round transcription reactions (Barker et al. 2001a; Rutherford et al. 2007). Supercoiled plasmid templates contained the *RNA1* promoter (used as a control) and either the *rrnB* P1 promoter (−66, +50) or the *argI* promoter (−45, +32). Transcripts were separated on gels and analyzed by PhosphorImaging. DksA (2 μM), ppGpp (100 μM), or both were included when indicated.

RNAP–promoter complex decay assays

Competitor-resistant complex lifetimes were measured as described previously by either nitrocellulose filter retention or

using in vitro transcription as a readout (Barker et al. 2001a; Rutherford et al. 2007). The competitor was heparin (10 μg/mL). Details are provided in the Supplemental Material and in the figure legends.

Localized Fe^{2+}-mediated cleavage of HMK-labeled DksA

Radiolabeled HMK-His_6-tagged DksA was incubated with RNAP at 37°C, mixed with freshly prepared 500 μM $(NH_4)_2Fe(SO_4)_2$ and 100 mM DTT for 15 min at 37°C, and quenched with loading buffer. Reactions were separated by PAGE. See the Supplemental Material for details.

Acknowledgments

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