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DksA: A Critical Component of the Transcription Initiation Machinery that Potentiates the Regulation of rRNA Promoters by ppGpp and the Initiating NTP

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Summary

Ribosomal RNA (rRNA) transcription is regulated primarily at the level of initiation from rRNA promoters. The unusual kinetic properties of these promoters result in their specific regulation by two small molecule signals, ppGpp and the initiating NTP, that bind to RNA polymerase (RNAP) at all promoters. We show here that DksA, a protein previously unsuspected as a transcription factor, is absolutely required for rRNA regulation. In Δ*dksA* mutants, rRNA promoters are unresponsive to changes in amino acid availability, growth rate, or growth phase. In vitro, DksA binds to RNAP, reduces open complex lifetime, inhibits rRNA promoter activity, and amplifies effects of ppGpp and the initiating NTP on rRNA transcription, explaining the *dksA* requirement in vivo. These results expand our molecular understanding of rRNA transcription regulation, may explain previously described pleiotropic effects of *dksA*, and illustrate how transcription factors that do not bind DNA can nevertheless potentiate RNAP for regulation.

Introduction

Ribosomal RNA synthesis constitutes the majority of transcription in moderate to rapidly growing bacteria, and its regulation has been investigated intensely since the earliest days of molecular microbiology (Sands and Roberts, 1952; Schaechter et al., 1958). In *Escherichia coli*, rRNA transcription is regulated primarily at the level of initiation from rRNA promoters (Gourse et al., 1996; Schneider et al., 2003b), although mechanisms also exist for ensuring efficient elongation of RNA polymerase (RNAP) on rRNA operons (Condon et al., 1995). rRNA transcription is the rate-limiting step in ribosome synthesis (Gourse et al., 1996).

Here we report that the 151 amino acid polypeptide DksA is absolutely required for regulation of rRNA expression. rRNA promoter activity in Δ*dksA* mutants fails to shut down upon entry into stationary phase, to increase after dilution of stationary phase cells into fresh medium, or to respond to changes in growth rate or to amino acid starvation. In conjunction with previous results, we suggest that *dksA* potentiates regulation of rRNA promoters in response to changing concentrations of two small molecule effectors, the initiating NTP and ppGpp.

E. coli contains seven rRNA operons, each transcribed from tandem promoters, *rrn* P1 and *rrn* P2. Most rRNA transcription initiates from the P1 promoters during rapid growth (Gourse et al., 1996), while the P2 promoters account for most rRNA transcription at low growth rates and in prolonged stationary phase (Murray and Gourse, 2004). Classical mechanisms of regulation of transcription initiation involve the action of activators and repressors, DNA binding proteins whose effects on RNAP are localized to different promoters by specific binding sites in the promoter region. Although the transcription factor Fis helps to recruit RNAP to *rrn* P1 promoters (Bokal et al., 1995), and the nucleoid protein H-NS can inhibit rRNA promoter activity (Afflerbach et al., 1998), most regulation of rRNA transcription is not a function of the action of classical activator or repressor proteins (Bartlett and Gourse, 1994; Murray et al., 2003b; Schneider et al., 2003b; Murray and Gourse, 2004).

Regulation of rRNA promoters results primarily from the action of the initiating NTP (iNTP) and the "alarmone" guanosine 5′-diphosphate 3′-diphosphate (ppGpp) that act on a kinetic intermediate, the open complex, whose lifetime is rate limiting for rRNA transcription initiation (Gourse, 1988; Barker et al., 2001b). rRNA promoters are unusually sensitive to the concentrations of these small molecules. When NTP concentrations increase, the iNTP transiently stabilizes the rRNA promoter open complex, most likely driving transcription initiation forward by mass action (Gaal et al., 1997; Schneider et al., 2002; Murray et al., 2003b; Murray and Gourse, 2004).

ppGpp is synthesized primarily by the ribosome-associated RelA protein in response to uncharged tRNAs in the ribosome's A site (Hogg et al., 2004). ppGpp binds to a site adjacent to, but not overlapping with, the active site of RNAP (Artsimovitch et al., 2004), and although its mechanism of inhibition of transcription initiation is not completely understood, ppGpp decreases the lifetimes of all open complexes, inhibiting transcription from promoters in which this complex is intrinsically short lived (Barker et al., 2001b; Murray et al., 2003b; Schneider et al., 2003b; Murray and Gourse, 2004). The effects of changing concentrations of ppGpp and NTPs on rRNA transcription can be observed in vitro using solution conditions that result in short-lived *rrn* promoter open complexes (Gaal et al., 1997; Barker et al., 2001b). These small molecule effectors control rRNA expression at different times in the bacterial growth cycle; their functions are not redundant (Murray et al., 2003b).

DksA was originally identified in *E. coli* as a multicopy suppressor of the temperature sensitivity of *dnaKJ* mutants (Kang and Craig, 1990). Deletion and overexpression of *dksA* have pleiotropic effects, including defects in chaperonin function, gene expression, cell division,

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amino acid biosynthesis, quorum sensing, and virulence (Kang and Craig, 1990; Bass et al., 1996; Turner et al., 1998; Webb et al., 1999; Ishii et al., 2000; Branny et al., 2001; Hirsch and Elliott, 2002; Brown et al., 2002). Previous studies have not elucidated the molecular mechanisms linking *dksA* and these phenotypes.

Two results prompted us to examine the effects of deletion of the *dksA* gene on regulation of rRNA promoter activity. First, complex amino acid polyauxotrophies are characteristic of strains unable to synthesize ppGpp (Δ*relA*Δ*spoT*) (Xiao et al., 1991), and *dksA* mutants also have complex amino acid requirements for maximal growth (Kang and Craig, 1990; Webb et al., 1999; see Discussion). Second, *Tn5* insertions in the *dksA* gene were the most frequent mutants obtained following mutagenesis of the *E. coli* chromosome and screening for specific increases in rRNA promoter activity (J. Lemke, B.J.P., and R.L.G., unpublished data).

We demonstrate here not only that *dksA* is essential for regulation of rRNA promoters in vivo, but also that purified DksA binds to RNAP in vitro, increases the rate of open complex decay at all promoters tested, and specifically inhibits transcription from rRNA promoters. We propose that DksA is needed to alter the kinetics of rRNA promoters so that they can be affected by the changes in NTP and ppGpp concentrations that occur in vivo. Our results, in conjunction with the high-resolution structure of DksA presented in the accompanying report (Perederina et al., 2004 [this issue of *Cell*]), demonstrate that DksA is a crucial component of the transcription initiation machinery and, more generally, illustrate how a small protein can potentiate regulation of specific promoters by small molecules that interact with all transcription complexes.

Results

rRNA Promoter Activity Increases in Δ*dksA* Mutants during Steady-State Growth

The effect of DksA on rRNA promoter activity in vivo was determined by comparing activities of *rrnB* P1 promoter-*lacZ* fusions in wild-type and Δ*dksA* strains grown in a variety of different media. In the wild-type strain, rRNA promoter activity increased with growth rate (Figure 1A). This characteristic growth rate-dependent regulation (Schaechter et al., 1958) was completely abolished in the mutant strain. Differences in promoter activity between the wild-type and mutant strains were observed under all conditions but were especially dramatic at the lowest growth rates (e.g., there was a 7-fold increase when comparing the Δ*dksA* and wild-type strains at the same growth rate or a 6-fold increase in the same medium; Figure 1B). Similar results were also obtained for *rrnD* P1-*lacZ* and *rrnB* P2-*lacZ* fusions and for *rrnB* P1 promoter-*lacZ* fusions with a variety of upstream endpoints (−46, −61, and −152; data not shown). Promoter activities were also assayed by primer extension with similar results (data not shown; see also below). Not surprisingly considering these effects on rRNA promoter activity, the Δ*dksA* mutant was most impaired at slow growth rates, where a negative regulator of rRNA transcription would be most needed to prevent overinvestment of the cell's energy in ribosome synthesis.

To control for potential effects of the Δ*dksA* mutant on *lacZ* message stability, translation, β-galactosidase activity, or on general promoter function, a *lacUV5* promoter-*lacZ* fusion was also tested (Figure 1C). This fusion was constructed using *lacUV5* promoter sequence extending only to +1, the transcription start site, so as to create a reporter making the exact same transcript as the *rrnB* P1-*lacZ* fusion. β-galactosidase activity from this fusion increased little, if at all, in the Δ*dksA* strain (Figure 1C). Thus, the effect of the Δ*dksA* mutation is promoter specific.

Total RNA levels include the cumulative effects of synthesis, processing, and decay of RNA, and stable RNA (rRNA and tRNA) makes up greater than 95% of the total RNA in the cell. Thus, RNA:protein ratios are indicative of bulk rRNA and tRNA expression. RNA:protein ratios were measured in the wild-type and Δ*dksA* strains in order to confirm both that the increase in rRNA promoter activity observed with the *lacZ* fusions in the Δ*dksA* strain reflected an increase in rRNA output and also that most or all of the rRNA operons were affected by loss of *dksA*. As observed with the rRNA promoter-*lacZ* fusions, RNA:protein ratios were dramatically increased at low growth rates, and rRNA growth rate dependence was abolished in the Δ*dksA* strain (Figure 1D). These results suggest that DksA affects the activities of most or all rRNA promoters.

Loss of the DksA Protein Is Responsible for the Effect of the Δ*dksA* Mutation on rRNA Expression

The *dksA* gene is located at about 3.5 min on the *E. coli* chromosome (Kang and Craig, 1990), just downstream from *sfsA* and upstream of *yadB*. Complementation assays were used to determine whether the loss of *dksA*, rather than an indirect effect on the flanking genes, was responsible for the increased *rrnB* P1 promoter activity and reduced growth in minimal medium observed in the Δ*dksA* strain. Plasmids expressing native DksA or a histidine-tagged version of DksA (HisDksA) eliminated overexpression from *rrnB* P1 completely and restored wild-type growth (Table 1), while plasmids expressing the upstream or downstream gene products were unable to do so. These results indicate that the phenotypes of the Δ*dksA* strain result from loss of the DksA protein, consistent with the results reported below obtained in vitro with purified HisDksA.

Changing Levels of iNTPs and ppGpp Are Not Responsible for Increased rRNA Promoter Activity in the Δ*dksA* Strain

To determine whether changes in the concentrations of ppGpp and NTPs were responsible for the loss of rRNA transcription regulation observed in the Δ*dksA* strain, these regulators were measured. The ppGpp concentration increased ~3.5-fold in the Δ*dksA* strain (consistent with a previous report; Brown et al., 2002), the ATP concentration decreased by ~40%, while the GTP concentration decreased ~10% (Supplemental Table S1 at http://www.cell.com/cgi/content/full/118/3/311/DC1). These changes could not account for the observed increase in rRNA promoter activity since increased concentrations of the negative effector, ppGpp, would be

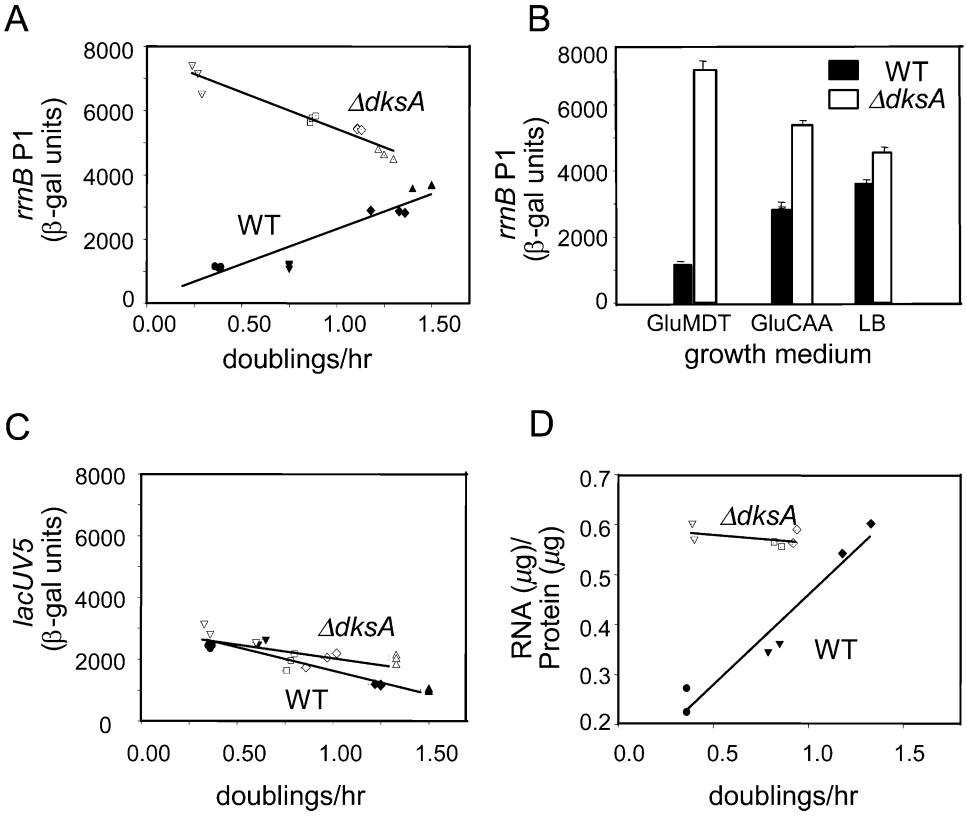

Figure 1. rRNA Is Overexpressed in a Δ*dksA* Strain during Exponential Growth

(A) *rrnB* P1 promoter activity (promoter sequence endpoints −46 to +1) as a function of growth rate. Wild-type, RLG4996 (closed symbols); Δ*dksA*, RLG6348 (open symbols).

(B) *rrnB* P1 promoter activities from (A) graphed as a function of growth medium.

(C) *lacUV5* promoter activity (*lac* promoter sequence endpoints −46 to +1). Wild-type, RLG5036; Δ*dksA*, RLG6346.

(D) RNA:protein ratios from the strains used in (A). rRNA promoter activity was assayed from single copy, chromosomal promoter-*lacZ* fusions in both wild-type and Δ*dksA* strains grown in M9 minimal medium supplemented with glycerol (●), glucose with methionine, aspartic acid, and threonine, GluMDT (▼), glycerol with casamino acids, GlyCAA (■), glucose with casamino acids, GluCAA (◆), or in LB (▲). The growth rates of the wild-type and mutant strains were similar at higher growth rates, but the Δ*dksA* mutant could not grow in M9 glycerol medium lacking amino acids. The growth rate of the Δ*dksA* strain in M9 glucose medium containing methionine, aspartate, and threonine (M9-glucose-MetAspThr) was roughly comparable to that of the wild-type strain in M9 glycerol medium lacking amino acids. Experiments were performed in duplicate or triplicate (indicated by multiple symbols), using at least two independent transductants.

expected to decrease, not increase, rRNA promoter activity and since decreased concentrations of the positive effector, the iNTP, would be expected to decrease, not increase, rRNA promoter activity (Murray et al., 2003b; Murray and Gourse, 2004). Furthermore, the effects of the Δ*dksA* strain do not appear to be mediated by two other proteins that affect rRNA transcription, Fis and H-NS (data not shown).

We interpret the observed changes in ppGpp and NTP concentrations as consequences of the overproduction of rRNA in the Δ*dksA* mutant. We suggest that overproduction of ribosomes leads to depletion of ATP/GTP

Table 1. Plasmid-Derived DksA Complements a Δ*dksA* Mutant

Strain/Plasmid	Relative β-Galactosidase Activity	Growth Rate (Doublings/hr)
Wild-type/vector (RLG4996/pRLG6332)	1	0.96 ± 0.00
Δ*dksA*/vector (RLG6348/pRLG6332)	4.85 ± 0.08	0.41 ± 0.02
Δ*dksA*/p*dksA* (RLG6348/pRLG6333)	1.12 ± 0.03	0.96 ± 0.00
Δ*dksA*/p*HisdksA* (RLG6348/pRLG7069)	0.85 ± 0.02	0.90 ± 0.00
Δ*dksA*/p*sfsA* (RLG6348/pRLG6335)	4.55 ± 0.16	0.49 ± 0.03
Δ*dksA*/p*yadB* (RLG6348/pRLG6334)	6.18 ± 0.25	0.49 ± 0.02

β-galactosidase activities in strains containing an *rrnB* P1-*lacZ* fusion, *rrnB* P1 sequence endpoints −46 to +1 (see Experimental Procedures and Supplemental Table S2 online). Cells were grown in MOPS with glucose (0.4%), methionine, aspartate, threonine (40 μg/ml each), and ampicillin (100 μg/ml). Averages are from three independent transformants.

and amino acids. Changing concentrations of NTPs and ppGpp have been implicated previously in feedback control of rRNA transcription (Gaal et al., 1997; Schneider et al., 2002; Schneider and Gourse, 2003). rRNA overexpression in the Δ*dksA* strain apparently overtaxes the ability of the feedback system to maintain homeostasis.

rRNA Promoter Activity Does Not Respond to Changes in Growth Phase or to Amino Acid Starvation in a Δ*dksA* Strain

We also measured the effects of the Δ*dksA* mutation on rRNA promoter activity during growth transitions, rather than in the steady-state situations shown in Figure 1. The mRNA produced from the *rrnB* P1-*lacZ* fusion has a short half-life, allowing estimation of instantaneous synthesis rates at specific times by primer extension (Murray et al., 2003a).

During entry into stationary phase, *rrnB* P1 activity in the wild-type background decreased ~40-fold from its log phase level (Figure 2A). We showed previously that changes in NTP and ppGpp concentrations together account for most of this response (Murray et al., 2003b). In contrast, the activity of the same promoter decreased only 4-fold upon entry into stationary phase in the Δ*dksA* background (Figure 2B).

Following dilution of stationary phase cells into fresh medium (outgrowth), *rrnB* P1 activity in the wild-type background increased rapidly (Figure 2C), consistent with previous observations (Murray et al., 2003b). A dramatic increase in ATP concentration is responsible for the rapid increase in *rrnB* P1 promoter activity (Murray et al., 2003b). In contrast, *rrnB* P1 activity did not increase rapidly during outgrowth in the mutant strain (Figure 2C).

ppGpp concentrations increase dramatically following amino acid starvation and negatively regulate rRNA promoter activity (stringent control). Addition of serine hydroxamate (to induce serine starvation) decreased *rrnB* P1 promoter activity ~12-fold by ~10 min in the wild-type strain (Figure 2D). However, *rrnB* P1 promoter activity decreased less than 2-fold in the Δ*dksA* mutant (Figure 2E). ppGpp concentrations increased both in the wild-type and Δ*dksA* strains, consistent with previous observations (Brown et al., 2002). Although the Δ*dksA* mutant accumulated somewhat less ppGpp than the wild-type strain, these concentrations of ppGpp should have been sufficient to inhibit rRNA promoter activity dramatically (Figure 2F; i.e., *rrnB* P1 was inhibited less than 2-fold in the mutant strain at 15 min, even though ppGpp concentrations were greater at this time than at 8 min in the wild-type strain when virtually complete inhibition of promoter activity was observed).

We conclude that in the Δ*dksA* strain, *rrnB* P1 is unable to respond to the changes in ppGpp and the iNTP levels that normally regulate rRNA transcription.

Purified DksA Directly and Specifically Inhibits Transcription from rRNA Promoters

The results above prompted us to determine whether DksA affects rRNA promoter activity directly. Purified HisDksA inhibited the wild-type *rrnB* P1 promoter ~20-fold (transcription was 0.05 ± 0.02 of that with buffer alone; Figure 3A, lanes 7 and 8), whereas HisDksA did not inhibit transcription from *rrnB* P1 (dis), a constitutive *rrn* P1 promoter mutant that has increased activity because it makes a long-lived open complex (Barker et al., 2001b; Barker and Gourse, 2001), or from two other unregulated promoters, *lacUV5* and *rna1* (Figure 3A, lanes 1–6). A completely unrelated histidine-tagged protein used as a control, thymidine kinase (HisTdk), had no effect on *rrnB* P1 activity (Figure 3A, lane 10). These results suggest that the effects observed in vivo (Figures 1 and 2) reflect direct inhibition of rRNA promoter activity by DksA.

DksA Decreases Promoter Open Complex Lifetime

Because several of the factors that regulate rRNA transcription do so, at least in part, by altering the lifetime of the open complex, we tested the effect of DksA on this step in transcription in vitro (see Figure 3 legend and Experimental Procedures). HisDksA decreased the half-life of the *rrnB* P1 open complex ~5.5-fold (Figure 3B). The magnitude of the effect on half-life was independent of promoter upstream endpoint or of the presence of the C-terminal domain of the α subunit (data not shown). The effect of HisDksA was also tested on the longer lived mutant *rrnB* P1 promoter, *rrnB* P1 (dis). HisDksA decreased the half-life of the *rrnB* P1 (dis) open complex about the same as for wild-type *rrnB* P1 (4- to 5-fold; Figure 3C). To quantify the effect of DksA on the *rrnB* P1 (dis) complex more precisely, we also measured DksA's effect on the complex assembled on a linear template (where the intrinsic half-life is much shorter), using the same solution conditions as for the wild-type promoter (Figure 3D). Under these conditions, HisDksA decreased the *rrnB* P1 (dis) lifetime ~5.5-fold, about the same as for the wild-type promoter. HisDksA also reduced the lifetime of the *lacUV5* open complex (Figure 3E) and the λP_L open complex (data not shown). These data suggest that DksA interacts with complexes formed on all promoters, but generally inhibits transcription only from those that form intrinsically short-lived open complexes.

DksA Amplifies Inhibition by ppGpp and Dependence on the iNTP Concentration In Vitro

rRNA promoters were virtually unresponsive to growth transitions in a Δ*dksA* mutant strain in vivo (Figure 2). The effect of DksA on open complex lifetime in vitro suggests a possible explanation for the lack of regulation of rRNA promoters in a Δ*dksA* strain. Under the solution and template conditions present in cells, DksA might be needed to alter the kinetics of rRNA promoters so that they can be affected by changes in the concentrations of NTPs and ppGpp. Since DksA is needed to potentiate regulation of rRNA promoters in vivo, we predicted that the magnitude of the effects of ppGpp and iNTPs observed in vitro might be amplified by the presence of DksA.

ppGpp inhibited *rrnB* P1 promoter activity ~2-fold in vitro in the absence of DksA, consistent with previous results (Figures 4A and 4B; see also Barker et al., 2001b). However, ppGpp decreased transcription ~20-fold in the presence of HisDksA. The concentration of ppGpp required for half-maximal inhibition was ~20 μM in the absence of HisDksA and ~1–2 μM in the presence of HisDksA (Figure 4B, inset), suggesting that DksA might

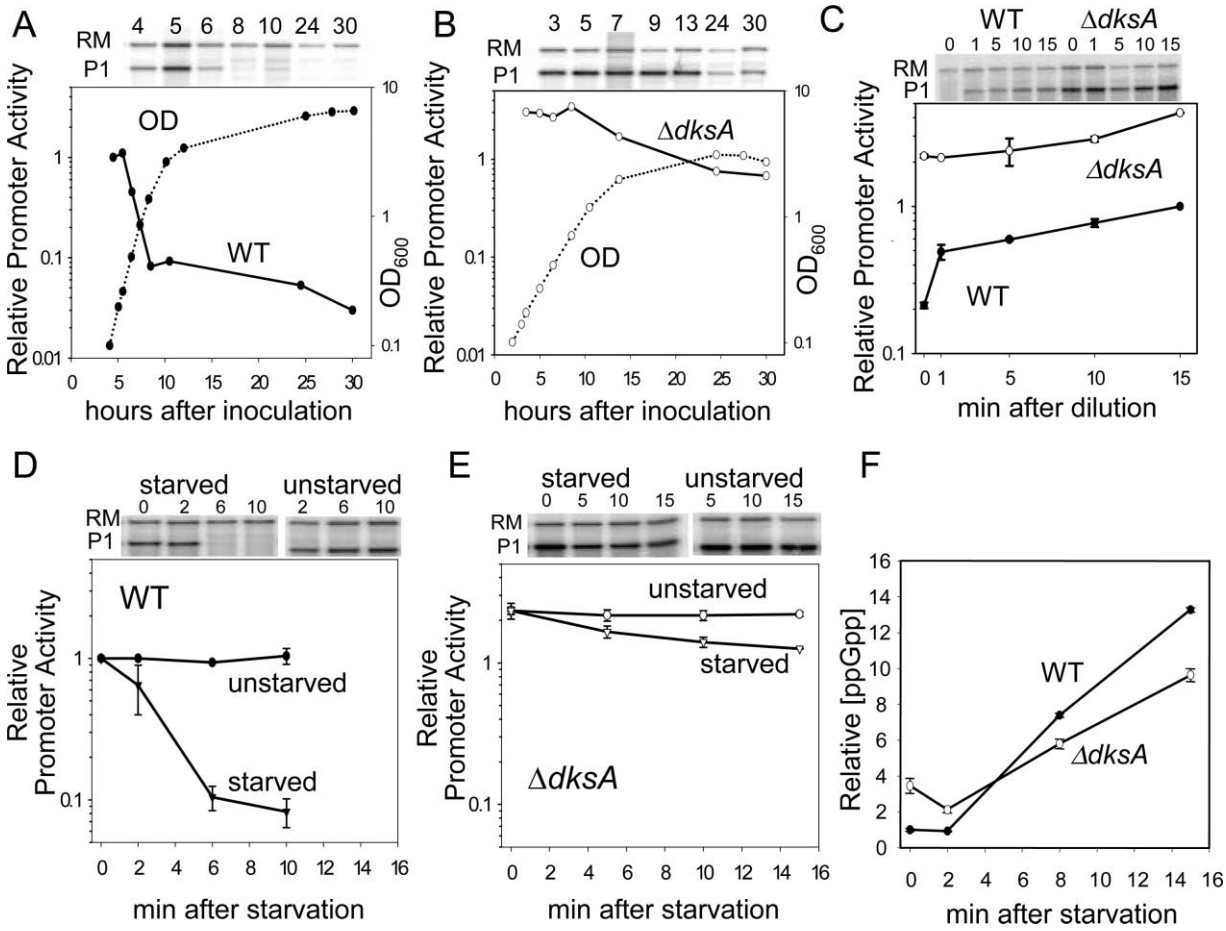

Figure 2. rRNA Promoter Activity Does Not Respond to Changes in Growth Phase or Amino Acid Starvation in a Δ*dksA* Mutant

rrnB P1 promoter activity was measured by primer extension. Having shown that sequences upstream of the −35 hexamer were not required for the effect of *dksA* on rRNA transcription in Figure 1, promoters were used that contained the *rrn* P1 UP element, thereby increasing RNA signal strength in primer extension experiments (*rrnB* P1 sequence endpoints −61 to +1). P1, transcript produced from *rrnB* P1-*lacZ* fusion; RM, recovery marker (see Experimental Procedures). Wild-type (closed symbols, RLG5950) and Δ*dksA* (open symbols, RLG7062) strains were grown in MOPS minimal medium supplemented with glycerol, casamino acids, and tryptophan. Band intensities plotted in the graphs (examples displayed above panels) were corrected for OD_{600} and volume.

(A and B) rRNA promoter activities during entry into and in extended stationary phase as a fraction of the activity in the wild-type 4 hr following dilution. The mutant strain did not achieve the same culture density as the wild-type, but it did reach a plateau, defining entry into stationary phase. Cultures were diluted into fresh medium to an OD_{600} 0.03 from colonies grown overnight on plates (containing the same medium), and samples were removed periodically for 30 hr, as indicated.

(C) rRNA promoter activity during outgrowth, measured by primer extension after dilution of stationary phase cells into fresh medium, normalized to the wild-type activity at 15 min after dilution. Error bars indicate the ranges obtained for two independent transductants of Δ*dksA*.

(D and E) *rrnB* P1 activity following amino acid starvation in wild-type and Δ*dksA* mutant, respectively. Cultures grew for ~4 generations to OD_{600} ~0.3 before addition of serine hydroxamate (final concentration 1 mg/ml) to induce starvation. The promoter activity measured by primer extension is the fraction of that in the wild-type at time 0. Error bars indicate the range from two independent experiments.

(F) ppGpp accumulation in the same culture conditions as in (D) and (E), except grown in the presence of ^{32}P-phosphate. ppGpp was extracted with formic acid and analyzed by TLC (see Experimental Procedures). ppGpp concentrations (averages from three independent cultures) are relative to those in the wild-type strain at time 0.

also directly stabilize the interaction of ppGpp with RNAP (see also Discussion and Perederina et al., 2004). As expected, ppGpp did not decrease transcription from the intrinsically long-lived *rna1* promoter either in the presence or absence of HisDksA. We suggest that DksA directly potentiates the effect of ppGpp on rRNA transcription in vivo.

HisDksA also increased the concentration of the iNTP required for half-maximal transcription from *rrnB* P1 ~3.5-fold in vitro (Figure 4C), a similar magnitude as the effect of DksA observed on open complex decay. These results are consistent with the model that DksA shifts the equilibrium away from the open complex, sensitizing rRNA promoters to the mass action effects of NTPs on transcription initiation. DksA thereby potentiates regulation of rRNA transcription by the changes in NTP concentrations that occur during growth transitions.

DksA Binds to Purified RNAP Core and Holoenzyme In Vitro

Since DksA inhibits rRNA promoter activity in a pure in vitro system, it must interact with promoter DNA, RNAP, or both. We did not detect either specific or nonspecific interactions between DksA and promoter

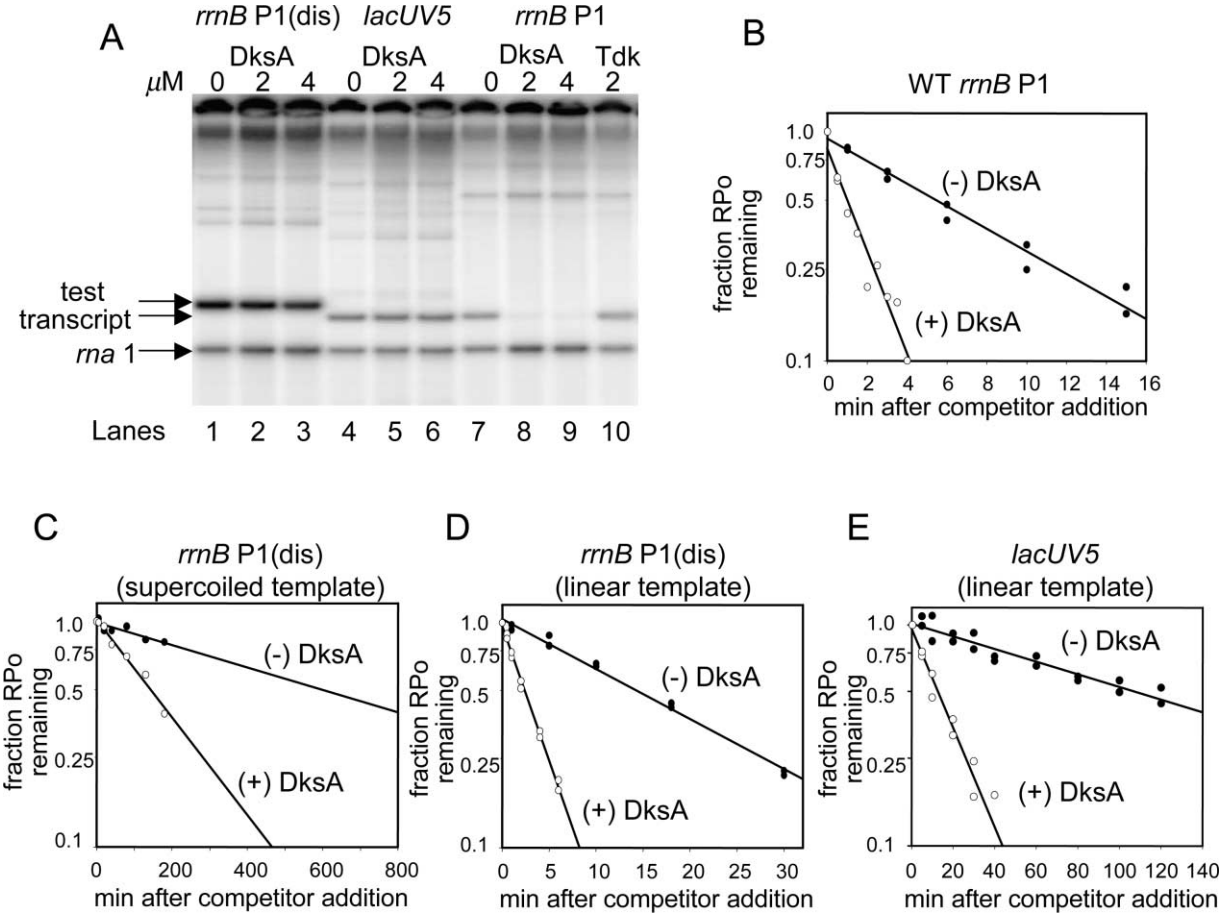

Figure 3. DksA Increases the Open Complex Decay Rate for All Promoters In Vitro but Specifically Inhibits Transcription from rRNA Promoters

(A) In vitro transcription. Single round transcription was performed on supercoiled plasmid templates ± HisDksA. Transcription buffer (see Experimental Procedures) also contained 50 mM NaCl, 500 μM ATP, 200 μM CTP and GTP, 10 μM UTP, and [α-^{32}P]UTP (2.5 μCi). Promoters in lanes 1–3, unregulated mutant promoter *rrnB* P1(dis) (−66 to +9; pRLG6120); lanes 4–6, *lacUV5* (−46 to +1; pRLG3422); lanes 7–10, wild-type *rrnB* P1 (−152 to +1; pRLG5943). Since DksA is not a DNA binding protein (see below) and since sequences upstream of the core promoter were not required to observe effects of *dksA* in vivo (Figure 1), the promoter endpoint used in these in vitro transcription assays is not crucial for the conclusion derived from the results (data not shown). *rrn* P1 promoters were chosen that contained the UP element to increase signal strength. Plasmid-encoded *rna* 1 transcript is indicated. Less than 200 nM DksA was sufficient for inhibition of *rrnB* P1 promoter activity in each of three assays.

(B, C, D, and E) Open complex decay. Lifetimes were determined using a transcription-based assay (B, C, and D) or a filter binding assay (E). (B), (C), and (D) contained 0.325 μM, and (E) contained 0.75 μM HisDksA. The transcription buffer contained 50 mM NaCl in (B) and (D), 70 mM NaCl in (C), and 110 mM KCl in (E). Templates: (B) supercoiled pRLG862 (*rrnB* P1 −88 to +1); (C) supercoiled pRLG6120 (unregulated *rrnB* P1(dis) promoter (−66 to +9); (D) 368 bp DNA fragment amplified by PCR from pRLG6120 (*rrnB* P1(dis) −66 to +9); (E) 190 bp fragment from pRLG4264 (*lac* sequences −59 to +38). The absolute decay rates differed slightly from experiment to experiment, but the ratio ± DksA was highly reproducible (≥ three assays).

DNA using footprinting or band-shift assays (data not shown). To determine whether there are direct interactions between RNAP and DksA, we bound HisDksA to a metal affinity resin and tested retention of RNAP or RNAP subassemblies (coimmobilization assay; Figure 5). Most RNAP holoenzyme was retained on the DksA column under low salt conditions and eluted only with a high salt wash (Figure 5A). In contrast, when an unrelated histidine-tagged control protein, HisTdk, was bound to the column, RNAP was not retained and appeared in the flowthrough (FT). RNAP lacking the σ subunit (core RNAP; Figure 5B) was also retained by HisDksA but not by HisTdk. In contrast, the $\alpha_2\beta$ subassembly was not retained by either HisDksA or HisTdk (Figure 5C), indicating that the β′ subunit is essential for DksA binding to RNAP. The accompanying paper provides strong independent evidence for direct binding of DksA to RNAP and proposes that a C-terminal helix in β′ helps mediate this interaction (see Discussion and Perederina et al., 2004).

DksA Protein Concentrations Remain Relatively Constant with Growth Phase and Growth Rate

DksA affected rRNA expression under all growth conditions tested (Figures 1 and 2), suggesting that DksA is present at all times in vivo. To obtain a more quantitative indication of whether the concentrations of DksA change with growth conditions, we assayed DksA protein levels using Western blots and an anti-DksA antibody. Consistent with previous results from *Salmonella*

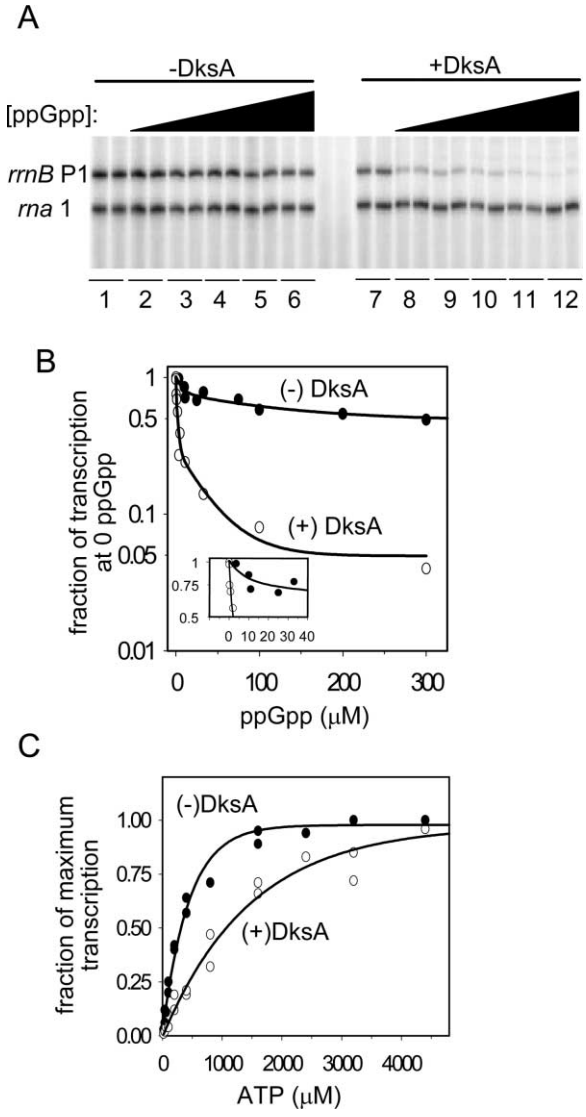

Figure 4. DksA Amplifies the Magnitude of Inhibition by ppGpp and the NTP Dependence of Transcription from *rrnB* P1 In Vitro

(A and B) Inhibition of *rrnB* P1 by ppGpp. Single round transcription was performed ± 0.19 μM HisDksA (a concentration with minimal effects on transcription by itself, allowing detection of synergistic ppGpp effects). Templates: supercoiled plasmid pRLG862, wild-type *rrnB* P1 (−88 to +1). Buffer: 50 mM NaCl and 500 μM ATP, 200 μM CTP and GTP, 10 μM UTP, and [α-^{32}P] UTP (2.5 μCi). Duplicate reactions for each ppGpp concentration: #1,7: 0 μM ppGpp; #2,8: 3 μM; #3,9: 10 μM; #4,10: 30 μM; #5,11: 100 μM; #6,12: 300 μM. (B) Results from two experiments, including (A). Closed circles: no HisDksA; open circles: 0.19 μM HisDksA. ppGpp concentration required for half-maximal inhibition was ~20 μM ± 0.0 in the absence of DksA and ~1 μM ± 0.2 in the presence of DksA (average and range from two experiments).

(C) ATP concentration dependence of multiple round transcription ±6.5 μM HisDksA (combined results from two experiments). Templates: supercoiled pRLG862, wild-type *rrnB* P1 (−88 to +1). Buffer: 165 mM NaCl, 200 μM GTP, 10 μM CTP and UTP, [α-^{32}P]UTP (2.5 μCi), and concentrations of ATP as indicated. (Low CTP concentrations were used to prevent nonphysiologically-relevant shifting of the transcription start site to −1 or −2 at high CTP:ATP ratios.) Transcripts were normalized to the maximal amount of trancription. Absolute ATP requirement for half-maximal transcription varied slightly from experiment to experiment, but relative NTP dependence ± HisDksA was highly reproducible.

typhimurium (Webb et al., 1999), DksA was present at similar concentrations at slow and fast growth rates in mid-log phase and in late stationary phase (Figure 6). We estimate that DksA is present at these times at ~3,000–10,000 copies per cell (data not shown). Thus, DksA likely potentiates the regulation of rRNA transcription under many (or all) conditions in vivo.

Discussion

DksA Interacts with Transcription Initiation Complexes and Potentiates the Regulation of rRNA Promoters

DksA was identified previously from multicopy suppression phenotypes or from effects of mutation(s) on a wide variety of cellular processes in vivo, but its direct molecular target was unclear. Here we have shown that DksA interacts with the transcription initiation machinery and plays a crucial role in control of rRNA synthesis. Figure 7 illustrates the role of DksA in the rRNA regulation circuitry. We propose that DksA potentiates the regulation of rRNA promoters by small molecules at least in part by reducing the lifetime of the open complex, bringing the half-life into the range where transcription can be affected by changes in the concentrations of ppGpp and iNTPs. It is possible that DksA potentiates regulation of rRNA transcription by the NTP concentration entirely through its effects on open complex lifetime. However, DksA likely also plays a more direct role in regulation by ppGpp (Figures 4A and 4B; see below and Perederina et al., 2004). Regulation of other promoters with similar kinetic characteristics, e.g., many tRNA promoters, might also require potentiation by DksA.

Our results suggest an explanation for differences in the magnitudes of the effects of ppGpp on rRNA promoter activity observed previously in vitro versus in vivo (e.g., 2- to 3-fold rather than the 10- to 20-fold inhibition observed in vivo; Barker et al., 2001b) or in cell extracts (e.g., Choy, 2000), and for the relatively low concentrations of iNTPs required for maximal rRNA promoter activity in some solution conditions in vitro (Gaal et al., 1997). Even in solution conditions that resulted in short-lived open complexes (e.g., using linear templates or buffers with high salt concentrations), inhibitory effects of ppGpp in vitro were always smaller than in vivo (Barker et al., 2001b). Furthermore, the concentrations of iNTPs saturating for transcription in vitro were somewhat lower than likely present in vivo (even using conservative estimates of the free ATP and GTP concentrations; Schneider and Gourse, 2004). Although inclusion of purified DksA in in vitro transcription reactions using physiologically relevant concentrations of the iNTP and ppGpp results in effects on transcription similar to those observed in vivo, we caution that the reaction reconstituted in vitro still may not completely mimic that present in cells with respect to solution conditions, template topology, or even the presence of additional protein components.

DksA Interactions with RNAP

We found that DksA interacts directly with RNAP, even in the absence of promoter DNA. The structure of *E. coli* DksA has recently been determined at 2 Å resolution

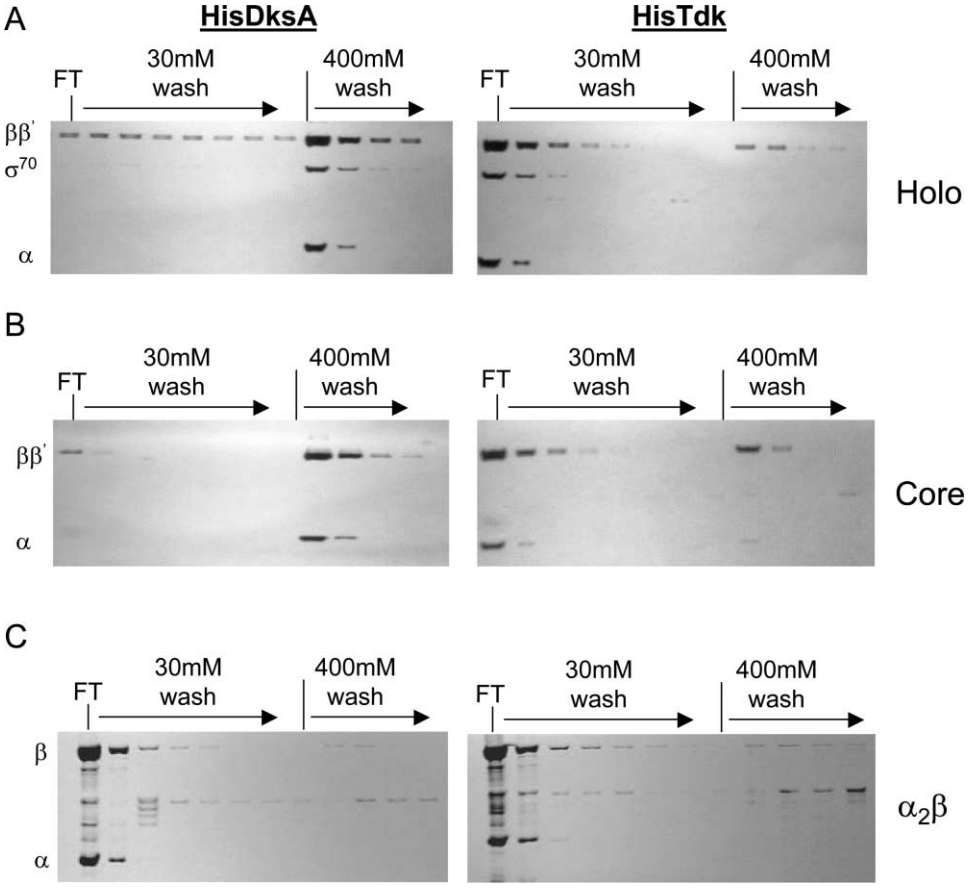

Figure 5. Immobilized DksA Binds to Purified RNAP Holoenzyme and to Core RNAP

Twenty to forty picomoles of (A) RNAP holoenzyme, (B) core RNAP, or (C) $\alpha_2\beta$ subassembly was added to columns containing metal affinity resin saturated with ~35 nmols HisDksA (left panels) or HisTdk (control, right panels). 0.5 ml fractions from the flowthrough (FT) and from low salt (30 mM NaCl) and high salt (400 mM NaCl) washes were TCA precipitated and visualized by PAGE and silver staining. In (A) and (B), most RNAP was retained by HisDksA, in contrast to (C), where most $\alpha_2\beta$ appeared in the FT. The β subunit in (C) was from inclusion bodies, which are not highly purified, accounting for the $\alpha_2\beta$-unrelated peptides retained by both HisDksA and HisTdk.

(Perederina et al., 2004). On the basis of the structure of DksA and biochemical experiments suggested by the structure, these authors propose that a coiled-coil domain in DksA extends deeply into the secondary channel of RNAP in a manner reminiscent of Gre factors. They further propose that two Asp residues at the tip of the coiled-coil coordinate a Mg^{2+} ion bound to ppGpp, thereby stabilizing the interaction of ppGpp with the transcription complex (see also Artsimovitch et al., 2004). Guided by the structural model described in the accompanying paper (Perederina et al., 2004), studies are underway to identify the interacting surfaces on RNAP and DksA that contribute to DksA function, to determine the molecular basis for the decrease in open complex half-life caused by DksA, and to further define DksA's role in interactions between RNAP and ppGpp.

DksA contains a zinc finger binding motif (Webb et al., 1999; Perederina et al., 2004). Using inductively coupled plasma mass spectrometry, we found that DksA binds one zinc atom per monomer (B.J.P. and R.L.G., data not shown), and a substitution for one of the cysteine residues within the zinc finger resulted in the same phenotype as a strain lacking the *dksA* gene (B.J.P. and R.L.G., unpublished data; Webb et al., 1999).

Relationship of the Role of DksA in rRNA Expression to the Pleiotropic Effects of *dksA* Mutants

Although we have not investigated the basis for the previously described pleiotropic effects associated with *dksA*, it would not be surprising if alterations in rRNA transcription had effects on the expression of products required for a wide variety of cell functions. The substantial overexpression of rRNA observed in Δ*dksA* mutants would be expected to titrate out RNAP, reducing expression from promoters limited by RNAP concentration. Such indirect effects of rRNA expression on amino acid biosynthetic promoters have been proposed as a partial explanation for the polyauxotrophies observed in mutants unable to synthesize ppGpp (Zhou and Jin, 1998; Barker et al., 2001a). In addition, overproduction of rRNA and/or specific tRNAs potentially could affect ribosome assembly, relative tRNA abundances, translation of specific mRNAs, and/or cellular reactions sensitive to ATP pools (which are altered in the Δ*dksA* strain; Supplemental Table S1 on the *Cell* website), with far-reaching effects on cell physiology.

Since DksA appears to be a part of the transcription initiation complex, it could potentially affect the activi-

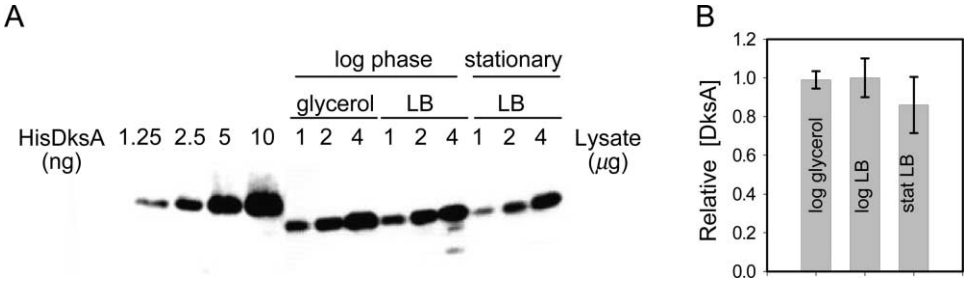

Figure 6. DksA Levels Remain Unchanged with Growth Rate and Growth Phase

(A) Western blot analysis of DksA from wild-type cells (RLG4996) in log phase in LB or M9 minimal medium plus glycerol (0.4%) or in in LB in stationary phase. A standard curve was produced using purified HisDksA (MW 20 kDa) for comparison to DksA (MW 17.5 kDa) from cellular lysates. The DksA band was not present in lysates from a cell lacking the *dksA* gene (data not shown). Polyclonal anti-DksA serum was a generous gift from D. Downs (University of Wisconsin).

(B) Western blots were quantified using ImageQuant (Molecular Dynamics), and DksA is graphed relative to the concentration in log phase cells in LB. Histograms represent averages from three independent experiments.

ties of other promoters directly. It has long been known that ppGpp increases transcription from certain amino acid biosynthetic promoters in vivo, but this effect has only been observed in vitro in the presence of cell extracts (e.g., Stephens et al., 1975; Choy, 2000). In the presence of DksA, however, ppGpp strongly stimulates transcription initiation from several amino acid promoters in a pure in vitro system (B.J.P, M.M.B., I. Kaganman, and R.L.G., unpublished data). The mechanism responsible for potentiation of ppGpp's effect on amino acid promoters is under investigation.

We emphasize that the physiological properties of Δ*relA*Δ*spoT* versus Δ*dksA* mutants are quite distinct both with respect to amino acid requirements and also many other phenotypes. These differences in phenotype likely result from complex defects and imbalances in the mutant strains and are consistent with our conclusion that DksA reduces the lifetimes of open complexes even in the absence of ppGpp; i.e., it does more than simply adapt RNAP to interact with ppGpp.

Results reported in two previous studies are consistent with our findings. First, Turner and colleagues noted

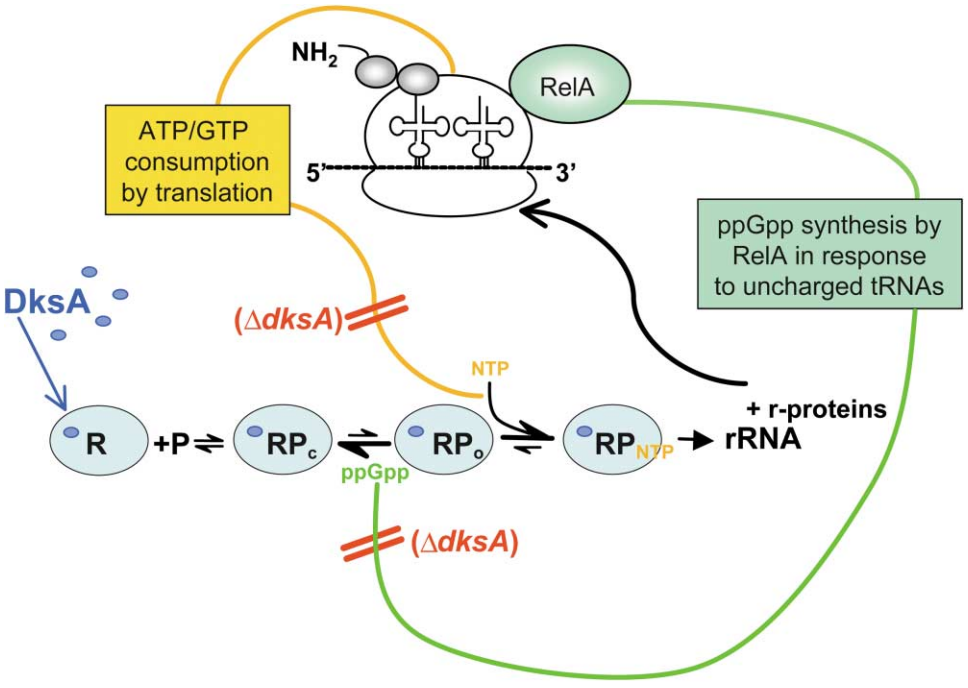

Figure 7. Model Depicting Contribution of DksA to Regulation of rRNA Expression

DksA (blue dots) binds to RNAP (light blue oval), decreases the half-life of the open complex (RP_o), thereby increasing the concentration of the iNTP (gold) required for transcription initiation, and amplifies inhibition by ppGpp (green). rRNA promoters are sensitive to changes in the concentrations of the iNTP and ppGpp because DksA brings the lifetime of the open complex into a range where it is rate limiting for transcription in vivo. Two feedback loops control rRNA transcription (Gaal et al., 1997; Murray et al., 2003b): ppGpp is synthesized by RelA in response to uncharged tRNAs in the ribosomal A site, and translation consumes ATP and GTP. In a Δ*dksA* mutant (red), the rRNA promoter open complex is longer lived, making it insensitive (double red slashes) to physiologically relevant changes in iNTP and ppGpp concentrations, eliminating regulation of rRNA transcription.

that *Salmonella typhimurium* Δ*dksA* mutants exhibited amino acid requirements for optimal growth and suggested that these amino acid requirements might indicate an involvement of DksA in the stringent response (Turner et al., 1998). Second, studies of the expression of the stationary phase sigma factor σ^S in a Δ*dksA* strain suggested a potential genetic link between *dksA* and ppGpp and indicated that mutations in *rpoBC* suppressed the amino acid requirements of a Δ*dksA* strain for maximal growth (Webb et al., 1999; Brown et al., 2002). A variety of *rpoBC* mutations mimic the effects of ppGpp by reducing rRNA promoter open complex lifetime, suggesting a basis for suppression of this Δ*dksA* phenotype (Bartlett et al., 1998; Zhou and Jin, 1998; Barker et al., 2001a).

Potentiation of Open Complexes by Small Proteins as a Strategy for Regulation

A short-lived open complex is a requirement for regulation of rRNA promoters by ppGpp and NTPs in vivo (Barker and Gourse, 2001; Schneider et al., 2003b). DksA apparently brings the half-life of the open complex into the range where it is sensitive to the variation in NTP and ppGpp concentrations present in cells. In principle, rRNA promoter sequences could have evolved to make intrinsically shorter lived open complexes, eliminating the requirement for DksA. However, we suggest at least three possible rationales for the requirement for DksA. First, DksA apparently has direct effects on interactions between RNAP and small molecules (e.g., ppGpp; see also Perederina et al., 2004). Second, DksA amplifies the effects of changes in the concentrations of small molecule signals. And third, although we have not identified natural conditions in which there are differences in the concentrations of DksA, inclusion of DksA in the control loops (Figure 7) provides a potential additional regulatory target. It is possible that the activity of DksA could vary in response to unknown signals. For example, since DksA is a substrate of the ClpXP protease (Flynn et al., 2003), it could potentially transduce signals mediated by that regulatory network.

In summary, regulation of rRNA expression is a situation in which selectivity does not derive from DNA sequences that specify binding of a transcription factor that can sense metabolites but rather from an alternative strategy in which a kinetic characteristic of the complex, determined by the DNA sequence of the core promoter, "localizes" regulation to specific promoters. The identification of DksA as a component of the transcription apparatus provides opportunities for additional layers of complexity that could contribute to the diversity of responses to nutritional and environmental signals that have evolved for regulating different promoters.

Experimental Procedures

Strains, Plasmids, and Protein Purification

Described in Supplemental Data and Supplemental Table S2 online.

β-Galactosidase Assays and RNA:Protein Ratios

λ monolysogens carrying promoter-*lacZ* fusions were grown at 30°C for ~4 generations in the media indicated in the figure legends. M9-based media contained 0.4% carbon source, 0.4% casamino acids with 40 μg/ml tryptophan, or 40 μg/ml of individual amino acids. At OD_{600} ~0.35, cells were harvested, placed on ice for 20 min, and lysed by sonication. RNA and protein levels were quantified as described (Gaal and Gourse, 1990), and β-galactosidase activity was determined by standard methods (Barker et al., 2001b).

Nucleotide Extraction

Wild-type and Δ*dksA* strains were grown as indicated in legends for Figure 2 and Supplemental Table S1. Nucleotides were extracted with formic acid and quantified as described previously (Schneider et al., 2003a; Schneider and Gourse, 2004). The identities of ATP, GTP, and ppGpp were verified by co-chromatography with commercial preparations of individual nucleotides (Pharmacia, Inc; Tri-Link, Inc.), visualized by UV shadowing.

RNA Extraction and Primer Extension Assays

Wild-type and Δ*dksA* strains containing promoter-*lacZ* fusions were grown at 30°C in MOPS minimal medium supplemented with 0.4% glycerol, 0.4% casamino acids, 40 μg/ml tryptophan, and 10 μg/ml thiamine (see figure legends). Boiling lysis extraction of RNA and reverse transcription were performed as described (Schneider et al., 2002). An RNA with the same primer binding site as the test RNA, but of a different length, was added at the time of lysis as a recovery marker (RM). Normalization to the RM allowed correction for differential losses between samples that potentially could occur at subsequent steps in the procedure.

In Vitro Transcription Assays

Multiple and single round in vitro transcription assays were performed using 10 nM RNAP ($E\sigma^{70}$), a generous gift from R. Landick (University of Wisconsin, Madison, Wisconsin). One nanomolar supercoiled plasmid was preincubated with RNAP and either HisDksA or HisDksA storage buffer (see above) for 10 min at 30°C in 40 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 0.1 μg/μl BSA, along with NaCl and NTPs at the concentrations indicated in the figure legends. Transcription reactions were initiated by the addition of NTPs. For single round assays, 200 nM of a 60 nt double-stranded DNA fragment containing the *full con* promoter (Gaal et al., 2001) was added with the NTPs as a competitor to prevent reinitiation. After 10 min, reactions were stopped with an equal volume of formamide loading buffer, electrophoresed on 7 M urea-6% polyacrylamide gels, then visualized and quantified by phosphorimaging. Buffer conditions were always the same when comparing effects of DksA and/or other factors on the same promoter but were adjusted between different promoters or for different kinds of assays (e.g., 50 mM, 70 mM, or 165 mM NaCl, supercoiled versus linear template, transcription versus half-life measurement) so as to put the absolute lifetime into the experimentally relevant range.

Open Complex Half-life Assays

Open complex half-life was determined using a transcription-based assay essentially as described (Barker et al., 2001b). 325 nM HisDksA or HisDksA storage buffer (see above) was preincubated with 1 nM supercoiled plasmid (or linear PCR product) and 10 nM RNAP for 10 min at 30°C in the transcription buffer described above. At time 0, 200 nM double-stranded DNA was added as a competitor (as described above), and at different times, 10 μl aliquots were added to tubes containing 1.25 μl NTPs (final concentration 500 μM ATP, 200 μM CTP and GTP, 10 μM UTP, and [α-^{32}P] UTP (2.5 μCi). Transcription reactions were stopped after 10 min and analyzed as described above. In Figure 3E, half-life was measured using a filter binding assay (Barker et al., 2001b). The DNA fragment, ~0.2 nM ^{32}P-end labeled 190 bp EcoRV-BglII restriction fragment derived from pRLG4264 (−130 to +60 with respect to the *lacUV5* transcription start site), was preincubated with RNAP (10 nM) in 10 mM Tris-Cl, 110 mM KCl, 100 μg/ml BSA, 1 mM DTT, and 10 mM $MgCl_2$ for 5 min at 30°C. HisDksA (750 nM) was added for 5 min, then heparin (10 μg/ml) was added, samples were removed at the indicated times and filtered, and open complexes were quantified as described previously.

Coimmobilization Assays

Metal-affinity resin (Talon, Clontech) was loaded onto 10 ml Polyprep columns (Bio-Rad) and washed with 40 bed volumes of 40 mM

Tris (pH 7.9), 10 mM $MgCl_2$, 5% glycerol, 30 mM NaCl, and 3 mM 2-mercaptoethanol (wash buffer). Approximately 35 nmol HisDksA or HisTdk was bound to the resin and washed with 80 vol wash buffer. Approximately 20–40 pmol purified RNAP holoenzyme, purified core RNAP (a generous gift from R. Landick), or an $\alpha_2\beta$ subassembly (reconstituted using purified α subunits and β subunit from inclusion bodies) was layered on top of the resin and allowed to flow through by gravity. The column was washed with 16 vol wash buffer and then 8 vol high salt buffer (wash buffer + 400 mM NaCl). Fractions (0.5 ml) were collected, TCA precipitated, resuspended in loading buffer (Invitrogen), separated on 10% polyacrylamide gels (Invitrogen), and visualized by silver staining.

Western Blot Assay

Wild-type cells (RLG4996) were inoculated from overnight plates (starting OD_{600} ~0.025), grown for ~4 generations for log phase cultures (to OD_{600} ~0.4) and for 24 hr for stationary phase cultures (OD_{600} ~5–6), then placed on ice for ~30 min. Cultures were lysed by sonication, centrifuged, and total protein concentration in soluble fractions was determined using the Bradford Assay Reagent (Bio-Rad), using BSA as a protein standard. Protein lysates were separated on 4%–12% polyacrylamide gels (Invitrogen), transferred to PVDF membranes (Bio-Rad) by electrophoresis, and the Western blot was performed using standard procedures (Bio-Rad) and a rabbit polyclonal anti-DksA serum (kindly provided by D. Downs; University of Wisconsin; precleared with a total cell lysate from a strain lacking *dksA* [RLG6348]), an HRP-conjugated secondary antibody (Santa Cruz Biotechnology), and SuperSignal West Pico chemiluminescent substrate (Pierce).

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