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Role of the coiled-coil tip of *Escherichia coli* DksA in promoter control

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Abstract

E. coli DksA works in conjunction with the small molecule ppGpp to regulate transcription initiation negatively or positively, depending on the identity of the promoter. DksA is in a class of transcription factors that do not bind directly to DNA like classical repressors or activators but rather bind in the RNA polymerase (RNAP) secondary channel like the transcription elongation factors GreA and GreB in *E. coli* and TFIIS in eukaryotes. We found that substitution for either of two residues in its coiled-coil tip, D74 or A76, eliminates DksA function without affecting its apparent affinity for RNAP. The properties of DksA-Gre factor chimeras indicated that the coiled-coil tip is responsible for the DksA-specific effects on open complex formation. A conservative substitution at position 74, D74E, resulted in a loss of DksA function in both negative and positive control, and an E44D substitution at the analogous position in GreA resulted in a gain of function in both negative and positive control. That a single methylene group has such an extraordinary effect on these transcription factors highlights the critical nature of the identity of coiled-coil tip interactions with RNAP for open complex formation.

Keywords

RNA polymerase; transcription initiation; secondary channel; ppGpp; transcription factor; open complex formation

Introduction

The *E. coli* genome encodes more than 300 proteins predicted to regulate gene expression, most of which are sequence-specific DNA-binding proteins.[1] The DNA sequences of the binding sites ensure that the effects of the regulators, either positive or negative, are targeted to specific promoters.

However, there are some transcription factors that interact directly with RNAP rather than with DNA.[2] For example, the 151 amino acid protein DksA binds directly to the secondary channel of RNAP and affects transcription by modifying the kinetic properties of the promoter complex.[3,4] DksA directly inhibits transcription from a large number of promoters, including promoters for rRNAs, many tRNAs and r-proteins, flagella, Fis, and DksA

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itself,[3, 5–10], and it activates transcription at many others, including promoters for the synthesis of amino acids and/or their transport, some virulence factors, the mRNA binding protein Hfq, and some promoters transcribed by the alternative sigma factor σ^E.[11–14]

DksA regulates transcription initiation in conjunction with changing concentrations of the small molecules guanosine 5′-diphosphate 3′-diphosphate and guanosine 5′-triphosphate 3′-diphosphate (collectively referred to here as ppGpp) and with changing concentrations of the transcript's initiating nucleotide (iNTP).[3] DksA levels are relatively constant,[3,15] but the levels of NTPs and ppGpp vary dramatically with changing growth rates, growth phases, and/or nutritional upshifts and downshifts.[16] Thus, DksA modifies the kinetic properties of the RNAP-promoter complex in response to growth conditions by making the promoter susceptible to changing NTP and/or ppGpp concentrations.[10,15]

The x-ray structure of DksA indicates that it has two domains, a long coiled-coil with two aspartic acidic residues at the tip and a globular domain containing a zinc finger motif.[4] The coiled-coil is similar in structure (although not in sequence) to that of two transcription elongation factors, GreA and GreB, that bind in the secondary channel of RNAP.[17–19] GreA and GreB crystal structures also have been solved at high resolution.[20,21] A low resolution cryo EM structure of GreB bound to *E. coli* RNAP[18] and a high resolution X-ray structure of another secondary channel binding protein, Gfh1, bound to *T. thermophilus* RNAP[22] have provided general models for how DksA might interact with RNAP. However, the details of DksA's interactions with RNAP remain unclear.

GreA cannot reduce the lifetime of the $E\sigma^{70}$ RNAP-promoter complex or inhibit transcription initiation *in vitro* and *in vivo* like DksA, whereas GreB can reduce the lifetime of the promoter complex and inhibit transcription initiation *in vitro* as well as *in vivo* when overproduced to compensate for its low concentration.[15] Neither GreA nor GreB can activate transcription initiation in conjunction with ppGpp like DksA.[15] In contrast, DksA cannot rescue paused transcription elongation complexes like the Gre factors. Gre factors enhance RNAP's intrinsic ability to cleave off the 3′ end of nascent RNA when it becomes disengaged from the active site and RNA backtracks into the enzyme's secondary channel.[4,19]

Here we identify amino acid residues required for DksA to perform positive and/or negative control of transcription initiation. Our studies show that the identity of a single aspartic acid residue in the coiled-coil tip of DksA is key for distinguishing its functions from those of GreA. Conversion of a glutamic acid residue at the coiled-coil tip of GreA to an aspartic acid residue, i.e. removal of a single methylene group, is sufficient to confer DksA-like activities on GreA in open complex formation *in vitro*. These results have important mechanistic implications for the function of transcription factors that bind in the RNAP secondary channel.

Results

Only one of the two conserved aspartic acid residues at the coiled-coil tip of DksA is critical for DksA function *in vitro* and *in vivo*

DksA has two aspartic acid residues, D71 and D74, at the tip of its coiled-coil (Fig. 1A). Previously, it was reported that a double substitution for these two residues, D71N/D74N, abolished DksA's ability to modulate transcription.[4,23] However, it was unclear whether one or both of these residues was required and whether these substitutions affected binding to RNAP or another function. To dissect the individual roles of these residues on DksA binding and function, we prepared variants in which each aspartic acid residue was mutated to asparagine, DksA-D71N or D74N. Single round transcription was performed using a

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supercoiled plasmid template containing the *rrnB* P1 promoter (endpoints −61/+1) fused to the terminator region from the *rrnB* operon to produce a ~150 nt transcript. Transcription reactions contained increasing amounts of DksA, either in the absence or presence of ppGpp. Wild-type and DksA-D71N inhibited transcription from *rrnB* P1 in a concentration-dependent manner (Fig. 1B, 1E), but DksA-D74N inhibited transcription much less, even at very high concentrations of the factor (maximum of 17% inhibition; Fig. 1C, 1F).

DksA greatly amplifies the effect of ppGpp on inhibition of rRNA transcription.[3] Both wild-type and DksA-D71N worked synergistically with ppGpp (10 to 20-fold inhibition at the highest DksA concentration; Fig. 1B, 1E), whereas DksA-D74N amplified the inhibitory effect of ppGpp much less (< 2-fold inhibition; Fig. 1D,1F).

The asparagine substitutions for D71 and D74 were chosen because they retained the length of the native aspartate side chain but changed the charge. We next asked whether the loss of function observed with DksA-D74N was also observed when the aspartic acid residue was replaced with a glutamic acid, maintaining the negative charge. As with DksA-D74N, D74E was defective for inhibition of *rrnB* P1 even with ppGpp, although it functioned slightly better than DksA-D74N (~4-fold inhibition; Fig. 1C, 1D, 1G). Thus, an amino acid side chain of the same charge as, and only slightly larger size than, D74 was insufficient for DksA function.

Because there was such a profound requirement for D74 in DksA function, we also examined the role of a nearby residue, A76. This alanine, two positions C-terminal to the second acidic residue in the coiled-coil tip (Fig. 1A), is conserved among several factors that bind in the RNAP secondary channel (e.g. GreA, GreB, and TraR).[17,24] An A76T substitution had emerged from a screen for dominant negative DksA mutants (J.- H. Lee, W. Ross, and R.L. Gourse, unpublished data). Like the D74N and D74E substitutions, the A76T substitution greatly reduced DksA's ability to inhibit transcription from *rrnB* P1, even in the presence of ppGpp (only ~3-fold inhibition; Fig. 1C, 1D, 1H).

The DksA substitution variants bind to RNAP

A possible explanation for the loss of DksA function in the D74 and A76 variants would have been that the purified proteins failed to bind to RNAP. Therefore, we measured the apparent affinities of DksA-D74N, D74E, and A76T for RNAP, compared to wild-type and DksA-D71N, using a previously-developed localized iron-mediated cleavage assay [25] that employs ^{32}P-labelled N-terminally HMK-tagged DksA. In this assay, Mg^{2+} in the active center of RNAP is replaced with Fe^{2+}, resulting in production of hydroxyl radicals that cleave the coiled-coil tip of DksA and its variants. The fraction of DksA cleaved was measured as a function of the RNAP concentration and normalized to the fraction cleaved at saturating RNAP concentration, allowing estimation of the apparent affinity of RNAP for DksA and its variants. Binding measured by this assay not only requires that DksA interacts with RNAP, but also that the coiled-coil tip approaches the RNAP active site. The apparent binding constant therefore likely reflects a physiologically relevant conformation of the complex (see also Materials and Methods).

Consistent with the results of our previous studies,[23,25] wild-type DksA bound to core RNAP and holoenzyme with similar apparent affinity, as did DksA-D71N ($K_{d\ app}$ ~100 nM, Table 1; Fig. S1). DksA-D74N, D74E, and A76T bound to RNAP core and holoenzyme with similar or even slightly higher apparent affinity than wild-type and DksA-D71N. We conclude that the defects in function of DksA variants D74N, D74E, and A76T do not result from misfolding of the mutant proteins and therefore reduced binding to RNAP but rather from effects on step(s) in their mechanisms of action after binding to RNAP.

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Interestingly, maximum Fe^{2+}-mediated DksA cleavage (plateau of the binding curve) varied for the different DksA variants (Table 1), but it did not correlate with apparent RNAP binding affinity. We infer that the efficiency of cleavage by hydroxyl radicals generated at the RNAP active site is an indicator of the local conformation of the coiled-coil tip region which, in turn, is affected by amino acid sequence, but the extent of cleavage, by itself, is not a quantitative measure of DksA binding affinity and/or function.

Effects of DksA coiled-coil tip substitutions on control of *rrnB P1 in vivo*

We next evaluated the activities of the mutant DksA proteins *in vivo* by measuring their abilities to complement a *dksA* deletion mutant strain (Δ*dksA*) (Fig. 2). As measured by Western blots (top of each panel), DksA protein levels were similar for the wild-type and DksA variants. DksA function was monitored from the β-galactosidase activity produced by an *rrnB* P1 promoter-*lacZ* fusion (bars in histogram). As observed previously,[3,15] *rrnB* P1 promoter activity was higher in log phase (~3.6-fold) in a strain lacking *dksA* and containing pINIII, the empty plasmid vector, compared to the Δ*dksA* strain containing pDksA, a plasmid producing the wild-type protein from the IPTG inducible promoter *lpp-lac* under the same induction conditions (Fig. 2A, compare columns 1 and 2). DksA-D71N (from plasmid pD71N) reduced *rrnB* P1 activity to almost the same level as wild-type pDksA (compare bars 1 and 3), consistent with its function *in vitro* (Fig. 1). In contrast, but consistent with the results *in vitro* presented in Fig. 1, expression of DksA-D74N, D74E, or A76T did not result in wild-type levels of *rrnB* P1 transcription (Fig. 2A, compare bars 4–6 with bar 1). In stationary phase (Fig. 2B), the β-galactosidase activities followed the same pattern as in log phase: expression of DksA-D74N, D74E, or A76T was unable to compensate fully for the absence of the *dksA* gene (compare bar 1 with bars 4–6). The fold-increases in *rrnB* P1 promoter activity caused by the loss of *dksA* were greater in stationary phase than in log phase, as has been reported previously for *rrnB* P1 and other promoters. [8,9,16] The effect of the loss of *dksA* is exacerbated in stationary phase, because DksA alters transcription in conjunction with changing concentrations of ppGpp and the iNTP, and there are large changes in the concentrations of these nucleotides when cells enter stationary phase. [16]

The DksA-D74N, D74E, and A76T variants are defective in positive control

ppGpp/DksA can activate transcription directly from a variety of promoters, including some responsible for amino acid biosynthesis and/or transport (positive control; see Introduction). Consistent with the defects of the DksA-D74N, D74E, and A76T proteins in inhibiting the rRNA promoter, the DksA variants were also defective in co-activating transcription from the *hisG* promoter (Fig. 3A, 3B), whereas DksA-D71N retained the majority of its ability to co-activate transcription. Qualitatively similar results were obtained with three other amino acid biosynthesis promoters, *livJ*, *argI*, and *thrABC*: DksA-D74N, D74E, and A76T were unable to co-activate transcription (Fig. S2).

DksA-D74N, D74E, and A76T are defective in reducing promoter complex lifetime

DksA and ppGpp do not block binding of RNAP to negatively-regulated promoters such as *rrnB* P1. Rather, they inhibit the progression from the closed complex to the open complex.[3,26] DksA and ppGpp together also directly activate some promoters, again by affecting step(s) after initial RNAP binding.[11] [Although DksA/ppGpp stimulates transcription from some promoters *in vivo* by increasing binding of RNAP, these effects are indirect, resulting from inhibition of the highly transcribed rRNA operons and redistribution of the RNAP from these operons to weak promoters].[11]

DksA reduces the lifetime of promoter - RNAP complexes at all $E\sigma^{70}$ - dependent promoters that have been examined. We have proposed that this effect on complex lifetime might

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reflect DksA's ability to lower the free energy of some intermediate or transition state in the transcription initiation pathway.[11] Whether this reduction in free energy decreases, increases, or has no effect on transcriptional output depends on the intrinsic kinetic properties of the individual promoter.

Therefore, we tested whether the effects of the DksA-D71N, D74N, D74E, and A76T variants on transcription initiation correlated with their effects on promoter complex lifetime. Transcript formation from *rrnB* P1 was used as a measure of the fraction of complexes remaining at different times after addition of a competitor (consensus promoter DNA; see Methods and Ref. 27). Wild-type and DksA-D71N decreased the half-life of the RNAP-*rrnB* P1 complex, but DksA-D74N, D74E, and A76T had little or no effect (Fig. 3C), consistent with their effects on transcription initiation (Figs. 1, 3, S2) and with the model that the same conformational change affects transcription initiation and promoter complex decay.

Gre-DksA chimeras with DksA-like activities: negative control

The sequences of the respective coiled-coil tip regions of DksA and the Gre factors differ except that they each contain two acidic residues (D71 and D74 in DksA, D41 and E44 of GreA and GreB) and an alanine two positions from the second acidic residue (A76 in DksA, A46 in GreA and GreB) (Fig. 4A). In order to analyze the effects of the tip residues in the same protein context, we created DksA-Gre factor chimeras (Fig. 4E–H). To determine the appropriate joints for the fusions, we overlaid the models of their respective coiled-coils and chose positions where the structures appeared to overlap. Guided by these models, we then created constructs in which the N-terminal and C-terminal sections of the chimeras came from either DksA (residues 1–68 and 77–151) or a Gre factor (1–38 and 47–158), and the 8 amino acid tip regions (residues 69–76 in DksA or 39–46 in GreA or GreB) came from the other factor (primer sequences used for plasmid constructs are in Supplementary Table S2). We named the chimeras in which the body of the protein was from DksA and the tip region was from GreA or GreB "DA" and "DB", respectively (Figs. 4E, 4F), and we named the reciprocal chimeras in which the body of the protein was from GreA or GreB and the tip region was from DksA, "AD" and "BD", respectively (Figs. 4G, 4H).

Single-round transcription reactions with increasing concentrations of the chimeric and wild-type proteins were performed on *rrnB* P1. As observed in our previous studies,[3,15] DksA and GreB strongly inhibited transcription, and the effects were larger (> 20-fold) when ppGpp was present (Figs. 5B, 5C). In contrast, GreA decreased transcription ≤ 2-fold even when ppGpp was included in the reaction (Figs. 5A, 5C). To determine whether differences in binding to RNAP explained the lower effects of GreA than DksA or GreB, we compared their apparent affinities for RNAP using the binding assay described previously [25] (Table 1). GreA bound to RNAP significantly better than DksA (although with a weaker apparent affinity than GreB). Thus, the weaker effect of GreA than DksA on transcription initiation is not attributable to weaker binding to RNAP.

The effects of the chimeras in which the body of the protein derived from DksA correlated with those of wild-type GreA and GreB: DB strongly decreased transcription (~15 to 20-fold in the presence of ppGpp) whereas DA did not (~2-fold) (Fig. 5A–C). Thus, the inhibitory effects depended on the identity of the coiled-coil tip. Binding of the DB chimera to RNAP was comparable to that of wild-type DksA ($K_{d\ app}$ ~73 nM versus ~103 nM for wild-type DksA; Table 1), whereas DA bound about 3-fold more weakly (~295 nM) than wild-type DksA. Thus a defect in binding might explain part of its weaker effect on transcription.

Because D74 in DksA was essential for negative control of transcription initiation (Fig. 1), but Gre factors contain a glutamate at the analogous position (Fig. 4), we next created DA

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and DB chimeras in which E74 (from the GreA or GreB tip region) was converted to an aspartate (DA-E74D and DB-E74D). Because GreB and DB strongly inhibited transcription in the presence of ppGpp (Fig. 5B; Ref. 15), it was not surprising that DB-E74D/ppGpp also strongly inhibited transcription (Fig. 5C). In contrast, whereas GreA and DA had only small effects on *rrnB* P1, DA-E74D strongly and specifically inhibited *rrnB* P1 (~20-fold) (Figs. 5A, 5C). Thus, the removal of a single methylene group was sufficient to convert DA into a promoter-specific transcription inhibitor, reinforcing our conclusion that negative control of transcription is strongly dependent on D74 (at least in the context of the GreA and DksA tip sequences; Figs. 1 and 5). The affinity of the DA-E74D chimera for RNAP ($K_{d\ app}$ ~126 nM; Table 1) was actually weaker than that of wild-type GreA for RNAP ($K_{d\ app}$ ~42 nM), suggesting that the gain of function was not attributable to better binding to RNAP.

We next examined the chimeras in which the body of the protein was from the Gre factor (Fig. 5D). Both the AD and BD chimeras strongly inhibited *rrnB* P1, consistent with the model that the identity of the coiled-coil tip, and in large part D74, is responsible for transcription inhibition. AD and BD bound to RNAP with approximately the same apparent affinitities as the factors they derived from ($K_{d\ app}$ ~42 nM for GreA and ~51 nM for AD; ~17 nM for GreB and ~19 nM for BD; Table 1), indicating again that the gain of inhibition function by the AD chimera was not attributable to better binding to RNAP.

The results with the AD chimera suggested that it might be possible to convert wild-type GreA into an efficient inhibitor of *rrnB* P1 simply by changing glutamate 44 to an aspartate. However, the E44D variant of GreA strongly reduced transcription not only from *rrnB* P1 but also from all other promoters on the template (data not shown). Similar results were obtained with an E44D variant of GreB (data not shown). These promoter-nonspecific inhibitory effects of the E44D substitutions might be related to a 70% reduction in transcription elongation reported previously for a GreA-E44D variant.[17]

Therefore, to assess function of the GreA-E44D variant in the context of a promoter complex, we measured its effects on open complex stability, using a nitrocellulose filter binding assay that does not require RNA product formation. Wild-type DksA and GreA-E44D reduced complex lifetime, whereas wild-type GreA and DksA-D74E did not (Fig. 5E): removal of a single methylene group (the difference between a glutamate and an aspartate R-group) converted GreA into a factor that mimicked the effect of DksA on open complexes.

We also measured the effect of wild-type GreB and a GreB-E44D variant on promoter complex stability. As expected, since wild-type GreB functions as a specific inhibitor of *rrnB* P1 *in vitro* as well as *in vivo* when the GreB concentration is increased to match that of DksA,[15] both GreB and GreB-E44D decreased open complex lifetime. These results imply that there are context effects affecting coiled-coil tip function: in the context of the GreB tip, E44 functions in reducing open complex lifetime whereas in the context of DksA and GreA, E44/D74E does not.

Coiled-coil tip requirements for positive control of open complex formation

We showed previously that in the presence of ppGpp, DksA but not the Gre factors could stimulate transcription initiation from *hisG* and several other promoters for amino acid biosynthesis or transport (see also Figs. 3 and S2).[15] Although they all had affinities for RNAP at least as strong as DksA, the chimeras DA, DB, DA-E74D, DB-E74D, and AD were all unable to activate transcription (Figs. 6A, 6B, 6C, S4; Table 1). Strikingly, however, BD (the GreB chimera with the DksA tip) was able to activate transcription (Fig. 6C), although not quite as well as wild-type DksA (~2.1-fold versus ~3.8-fold). The ability of BD to perform positive control was also ppGpp-dependent.

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To assess whether the BD chimera could increase the activity of the *hisG* promoter *in vivo*, transcription from P*hisG* was examined by primer extension during a stringent response in Δ*dksA* cells containing plasmid-encoded wild-type DksA or BD (Fig. 6D). After addition of serine hydroxamate (SHX) to induce amino acid starvation and ppGpp synthesis, *hisG* transcription increased 2.2-fold in Δ*dksA* cells with plasmid-encoded wild-type DksA and 2.3-fold with plasmid-encoded BD. In contrast, the *hisG* transcripts did not increase in cells with plasmid-encoded GreB. These effects on transcription paralleled those obtained *in vitro* (Fig. 6A–C).

Because the DksA tip region imparted positive control function on GreB, we asked whether the E44D substitutions alone would be able to convert Gre factors into activators of transcription initiation. Because the GreA and GreB-E44D variants inhibited RNA product formation (see above), we measured their effects on open complex formation using filter binding assays. Consistent with our previous results on the *argI* promoter, DksA/ppGpp increased the overall rate of open complex formation (Fig. 7).[11] Although GreA/ppGpp and GreB/ppGpp had no effect, the GreA and GreB substitution variants, GreA-E44D (Fig. 7A) and GreB-E44D (Fig. 7B), each increased the rate of open complex formation in the presence of ppGpp at least as efficiently as DksA/ppGpp. Thus, deletion of a single methylene group from the side chain of E44 was able to convert GreA and GreB into factors that increase the rate of open complex formation. The importance of these results for understanding the mechanism of DksA function in transcription initiation is discussed below.

Discussion

In previous work, we showed that GreB (when overproduced) could compensate for some, but not all, of the activities of DksA,[15] and we proposed that DksA acts allosterically.[26] Differences in how the Gre factors and DksA interact with RNAP must account for the differences in their effects on transcription initiation. There are no high resolution structures of DksA – RNAP – promoter DNA complexes captured at the relevant step on the pathway to open complex formation. Therefore, genetic and biochemical approaches must be utilized to determine how binding of DksA affects open complex formation. The goals of the present work were to identify residues in DksA that are critical for its function and that distinguish its activities from those of the Gre factors, and to use this information to develop models for the specific interactions with RNAP that lead to the differences in DksA versus Gre factor function.

D74: a critical residue in the DksA coiled-coil tip for regulation of transcription initiation

DksA and the Gre factors have structural similarities in their coiled-coil domains, most notably two acidic residues in their coiled-coil tips. We show here that the identities of the residues at their coiled-coil tips are responsible for their functional specialization.

Table 1 summarizes our data. Only the more C-terminal of the two aspartic acid residues in the DksA tip region (D74) was required for DksA function in regulating transcription initiation. In contrast, the more N-terminal of the acidic tip residues in the Gre factors (D41) is critical for the RNA cleavage reaction needed for rescuing backtracked transcription elongation complexes.[17] A GreA – DksA chimera in which the only residues that derived from DksA were those in the coiled-coil tip (AD) identified the tip as the critical region needed for specific inhibition of transcription initiation, and a GreB – DksA chimera in which the only residues that derived from DksA were those in the coiled-coil tip (BD) identified the tip as the critical region needed for specific stimulation of transcription initiation.

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Addition of a single methylene group to D74 of DksA (D74E) eliminated DksA function as a positive or negative regulator, and removal of a methylene group at position E44 in the Gre factors (the position analogous to D74 in DksA) resulted in a gain of function in each case (i.e. the E to D substitution enabled GreA to affect promoter complex lifetime, enabled DA to inhibit transcription, and converted both Gre factors into positive regulators of open complex formation. We suggest that the effect of the methylene group likely is steric, since aspartate and glutamate side chains have the same charge. This is consistent with the observation that substitution of a longer side chain on a neighboring residue, A76T, also strongly interfered with DksA function.

Although D74 is essential for DksA function and distinguishes DksA from the Gre factors, we do not mean to imply that it is the only residue in DksA essential for function. DksA function is complicated and does not derive from the identity of a single amino acid. There are many other elements in the protein that contribute to DksA structure and activity.

What is the function of the DksA coiled-coil tip in the promoter complex?

We envision two general models for the role of the coiled-coil tip of DksA, and specifically residues D74 and A76, in regulating transcription initiation. In one model, the coiled-coil tip interacts with RNAP and alters its conformation, with a resulting effect on open complex formation (see below). In the other model, D74 and A76 affect the conformation of DksA itself, perhaps changing the fraction of time it is in the form that affects RNAP. Changes in conformation (as a function of pH) have been reported for another secondary channel binding factor, Gfh1.[28] We note that the two models are not mutually exclusive.

Consistent with the first model, we recently found that the coiled-coil tip of DksA interacts directly with the β′ trigger loop of RNAP (C.W. Lennon, W. Ross, and R.L. Gourse, unpublished data). Supporting the importance of this interaction in regulation by DksA, trigger loop substitutions at the position of the interaction (C.W. Lennon, W. Ross, and R.L. Gourse, unpublished data), or a deletion of the trigger loop entirely, [26] eliminated DksA function.

We suggest that the DksA coiled coil - RNAP trigger loop interaction stabilizes the trigger loop in a conformation that affects promoter - RNAP interactions (the first model). A structure of *T. thermophilus* Gfh1 in complex with *Thermus* RNAP [22] provides a precedent for such a model. It was proposed that Gfh1 causes RNAP to adopt an alternative conformation, the “ratcheted state”, in which the width of the nucleic acid-binding channel increases and the bridge helix is kinked, with concomitant changes in its N and C-terminal regions and the adjacent fork-loop and switch regions. It is possible that DksA stabilizes a conformation of RNAP similar to that proposed for the Gfh1-RNAP complex.

At *rrnB* P1, DksA blocks the transition from the closed complex, in which DNAse I footprint protection of the promoter extends from ~−60 to ~+1, to a complex in which protection extends further downstream (to ~+10).[26] We suggested previously based on genetic evidence that the altered conformation of RNAP resulting from the DksA-trigger loop interaction includes changes in the switch regions and the bridge helix.[26] The switches and the bridge helix interact with DNA directly in the vicinity of the transcription start site, and the switches also serve as a hinge for the RNAP clamp domain. Thus, the DksA-trigger loop interaction could inhibit the transition from the closed complex to the next intermediate complex by interfering with promoter interactions directly and/or by restricting the mobility of the clamp and thus its interactions with downstream promoter DNA. We suggest that DksA variants with longer side chains at residues D74 (D74E) and A76 (A76T) interfere with DksA interactions with the trigger loop, thereby preventing the conformation that ultimately results in changes in transcription initiation.

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Relative binding affinities

All of the non-functional DksA variants reported here bound to RNAP, and all but one appeared to bind at least as well as wild-type DksA (Table 1, Figs S1, S3, S4). Therefore, we consider it unlikely that the effects of the coiled-coil tip variants reported here result from changes in their affinities for RNAP. However, we note that the measurements reported in Table 1 represent only apparent affinities and were used for comparison of binding of the coiled-coil tips of DksA, the Gre factors, and their variants to RNAP. These apparent affinities do not reflect potential interactions between other parts of the factors and RNAP that could in theory occur in the absence of the tip interactions. Furthermore, these measurements were made in the absence of promoter DNA. We reported previously that DksA strongly favors binding to free RNAP and to closed complexes rather than to open complexes.[25,26] We suspect that the concentrations of DksA required for binding to closed complexes more closely approximate those needed for binding to free RNAP. The higher concentrations of DksA needed for function in the transcription assays compared to the binding assays might reflect the equilibrium between closed and open complexes in these experiments.

NTPs likely approach the RNAP active site through the secondary channel,[29] and DksA binds in the secondary channel. However, DksA can affect steps in initiation before NTP addition.[11,26] Therefore, the reported effects of DksA on increasing the NTP concentration requirement ("NTP sensing") [3,6] do not result entirely (or perhaps even in part) from reducing NTP access to the active site. It is also not intuitively obvious how restricting NTP access could activate transcription at some promoters. The identification of DksA mutants that bind with wild-type apparent affinity and yet fail to inhibit or activate transcription supports the conclusion that restriction of NTP access by DksA is insufficient to explain the mechanism of DksA action.

Other roles of the coiled-coil tip of secondary channel binding factors

It was reported previously that DksA variants lacking both of the acidic residues in the DksA tip are still capable of alleviating the growth inhibition caused by collisions between the transcription and the replication machineries.[30] By implication, the DksA coiled-coil tip interaction with the trigger loop may not be needed to alleviate the effects of these collisions. It was also reported previously that DksA and/or Gre factors are needed to rescue certain DNA repair-defective mutants.[31] Furthermore, the first (more N-terminal) acidic residue in the Gre factor tip, D41, is crucial for stimulating RNA cleavage and therefore for efficient transcription elongation,[17] but not for effects on initiation.

Taken together, these data suggest that different factors that bind in the secondary channel can perform distinct functions: some factors act on conformations of RNAP specific to the promoter complex, whereas others act on specific off-pathway intermediates that form in the elongation pathway. Nature has thus devised ways of using these factors for regulation of transcription initiation and for correcting problems in alignment of the 3′ end of the RNA with the RNAP active site during transcription elongation. The activities of the DksA and Gre factors, the substitution mutants, and the chimeric variants could be distinctly different in different kinds of arrested, backtracked transcription elongation complexes. Although we have focused here on structure-function analysis of the DksA-like activities of the substitution mutants and chimeras on transcription initiation, future studies could employ these DksA and Gre factor variants to determine specific sections of the proteins responsible for transcription elongation activities, including transcript cleavage.

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The domains of secondary channel binding factors can be mixed and matched

Our DksA-Gre factor chimeras demonstrate that the DksA tip can function in the context of globular domains from either DksA or Gre factors. In particular, the DksA tip converted GreA into a negative regulator (chimera AD) and GreB into a positive regulator (chimera BD) of transcription initiation, demonstrating that an RNAP binding domain with a completely different structure than the Zn^{2+}-containing fold found in wild-type DksA can direct the DksA coiled-coil tip to its functional location. A DksA-like protein without a Zn^{2+}-binding globular domain has been reported to exist in *Pseudomonas aeruginosa*.[32]

It seems likely that chimeric factors analogous to those described here exist in nature. Evolution may have devised biological solutions in other bacteria in which a variety of globular domains are genetically combined with a variety of secondary channel binding domains. Such globular domains potentially could bind different environmental or nutritional signal molecules and transmit their effects through the coiled-coil domain for regulation of specific promoters.

Materials and Methods

Bacterial strains, plasmids, and reagents

The genotypes and sources of the *E. coli* strains and plasmids used in this study are listed in Supplemental Table S1. Primers for construction of plasmids are listed in Supplemental Table S2. Unless otherwise stated, all strains are derivatives of MG1655. Strain constructions were done by standard transduction methods using phage P1*vir*, and plasmid were constructed using standard methods for mutagenesis and transformation.[33,34] All constructions were verified by antibiotic resistance, PCR, DNA sequencing, and/or phenotypic assays. ppGpp was obtained from TriLink, Inc.

Fragments encoding chimeric proteins were generated using a two step PCR procedure and inserted into plasmid pINIIIA at the XbaI and HindIII sites and pET28a or pET33b at the NheI and HindIII sites (Table S1). Templates for PCR encoded the gene for the main body of the chimera (*dksA*, *greA* or *greB*). In Step 1, fragments corresponding to either the N-terminus to the tip region, or the tip region to the C-terminus, were amplified in two separate reactions, carried out with a primer corresponding to either the N or C-terminus (primers 3561 or 3562 for *dksA*; 5503 or 5504 for *greA*, and 5505 or 5506 for *greB*; Table S2). The other primer contained the tip-encoding sequence followed by a sequence coding for part of the body of the chimera (e.g. for construction of chimera DA, primers 5479 and 5480; Table S2). In step 2, the fragments produced in Step 1 were combined with the N and C-terminal primers to amplify the chimeric gene.

Protein Purification

N-terminally hexahistidine-tagged wild-type or mutant DksA or Gre factor proteins were produced from phage T7 promoter constructs in a BL21(DE3) variant in which the *greB* and *dksA* genes were both inactivated,[15] and purified by Ni-agarose affinity chromatography as described.[3] For the Fe^{2+}-cleavage experiments, His6-DksA or GreA/B variants all contained HMK tags as well.[25] Native RNAP was purified by standard procedures.[35] Protein concentrations were determined with the Bradford assay reagent (BioRad) using BSA as a standard.

In vitro transcription

Single or multiple-round *in vitro* transcription was performed as described [3,11] and in the figure legends, using 50 ng supercoiled plasmid and 10 nM RNAP at 30°C in transcription buffer (40 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA), and the

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indicated salt concentrations. Single round and multiple round assays have been used interchangeably for examination of promoters that are inhibited or activated by ppGpp/DksA.[3,11] Here, single round assays were used for experiments with promoters that were inhibited by ppGpp/DksA, and multiple round assays were used for assays that were activated by ppGpp/DksA. For the single round assays, complexes were formed by incubation of RNAP and template DNA at 30°C for 10 min. NTPs (500 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM UTP, and 1.0 μCi of [α-^{32}P] UTP, final concentrations) were added simultaneously with 200 nM double-stranded full consensus promoter DNA as competitor for free RNAP.[36] After 10 min for transcription elongation, the reactions were stopped with an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). For multiple round assays, supercoiled plasmid DNA template (50 ng), ppGpp (100 μM) and/or DksA (2 μM) were incubated at 30°C for 10 min, transcription was initiated by addition of RNAP to 10 nM, terminated after 10 min by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), and transcripts were separated on 6% polyacrylamide gels containing 7 M urea. The level of RNA product was quantified by phosphorimaging using Image-Quant software and normalized to that by wild-type RNAP in the absence of factors. Open complex formation was measured using filter retention assays (see below) as a readout of promoter occupancy for variants that inhibited transcription.

Determination of apparent binding constants by localized Fe^{2+}-mediated cleavage

Hydroxyl radical cleavage of secondary channel binding factors (wild-type and variants) was used as a quantitative estimate of binding to RNAP by replacement of Mg^{2+} in the active center of RNAP with Fe^{2+} as described previously.[25] Briefly, HMK-His$_6$-tagged proteins were ^{32}P-labelled, and DksA–RNAP complexes were formed by incubation of 10 nM DksA and at least a three-fold molar excess of RNAP (400 nM). Cleavage products were separated from intact factor by electrophoresis, and the dried gels were analyzed by phosphorimaging. Binding curves were generated at different RNAP concentrations. Saturation was obtained with each DksA variant, allowing apparent K_ds to be determined by calculating the fraction of cleaved DksA/cleaved plus uncleaved DksA at each RNAP concentration, and normalizing to the plateau value (maximum cleavage) at each RNAP concentration, as described.[25] Binding curves, apparent K_ds, and standard errors were generated using SigmaPlot. This measurement of binding depends not just on binding *per se* but also on positioning of the coiled-coil in the secondary channel near the RNAP active center. Therefore, the value reports a minimum estimate of the binding affinity (apparent binding constant, $K_{d\ app}$). The fraction cleaved at saturation (plateau value) for DksA or chimeric variants differed, most likely reflecting the altered geometry and thus differential accessibility of the tip region to the hydroxyl radicals generated by Fe^{++} in the active site (see ref 25).

RNAP-Promoter Complex Lifetime Assay

Promoter complex lifetime was measured using a transcription–based assay with a supercoiled plasmid template containing the *rrnB* P1 promoter or using a filter binding assay with a DNA fragment containing the *lacUV5* promoter.[27] For the transcription-based assay, 10 nM RNAP was incubated with 50 ng supercoiled plasmid pRLG5944 containing the *rrnB* P1 promoter (promoter sequence endpoints −61 to +1) in transcription buffer (40 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 1 mM DTT, 0.1 mg/ml of BSA) containing 30 mM NaCl and wild-type or variant DksA proteins or storage buffer (no factor) at 30°C for 10 min. Transcription reactions were initiated with NTPs (500 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM UTP, and 1.0 μCi of [α-32P] UTP, final concentrations) at the indicated times after the addition of double-stranded DNA as a competitor (200 nM, final concentration). Reactions were stopped, and the RNA products were separated by electrophoresis and

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quantified as described above. The amount of transcription relative to that at the time of competitor addition was plotted as a function of time. Filter binding assays were used in experiments in which the factor inhibited NTP addition (Fig. 5E, see text). Promoter fragments containing the *lacUV5* promoter (endpoints −60 to +40 from pRLG4264) were used as described in the figure legend and Ref. 37.

Kinetics of open complex formation

The rate of promoter complex formation was measured by addition of RNAP to the *argI* promoter, essentially as described.[11] As discussed in the text, filter binding assays were used because the GreA and GreB E44D factor variants inhibited NTP addition (Fig. 7). Reactions were carried out at 30°C in the transcription buffer described above except that it contained 75 mM NaCl, 4 nM RNAP, a ^{32}P-end-labelled *argI* promoter DNA fragment, 100 μM ppGpp, and 0.2 μM of the indicated transcription factor. Data were fit to a single exponential [$cpm_{obs} = (cpm_{plateau})(1-e^{-K_{obs}* t})$], where $cpm_{plateau}$ is the fraction of counts retained at equilibrium.[11]

β-Galactosidase Activity Assays

Derivatives of RLG7238, a Δ*dksA::tet* strain containing an *rrnB* P1 promoter-*lacZ* fusion as a reporter, were transformed with a derivative of plasmid pINIII containing the indicated factor, expressed from a *lpp-lac* promoter. Cells were grown at 30°C in LB, 0.1 mM IPTG, and 100 μg/ml ampicillin for ~4 generations to an OD_{600} of ~0.4 (for log phase) or for ~24 h to an OD_{600} of ~5.0 (for stationary phase). At the indicated time, cells were chilled on ice for ~20 min, lysed by sonication, and β-galactosidase activity was determined by standard methods.[27] The number of independent cultures tested and standard deviations are reported in the figure legends.

Western blots

Relative DksA concentrations were determined from cultures grown under the conditions used for the β-galactosidase assays. Briefly, cells were grown to an A_{600} of ~0.4 for the log phase sample, 5 ml were harvested by centrifugation, the cells were suspended in 1 ml of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 5% glycerol, 0.1 mM PMSF, lysed by sonication and centrifuged to remove insoluble materials. Cells were harvested at an A_{600} of ~5.0 for the stationary phase sample, 0.5 ml were harvested, and subsequent steps were the same as for the log phase sample. Total soluble protein concentration was determined with the Bradford Assay Reagent using BSA as a standard. Approx. 5 μg of each protein lysate was separated on 4–12% SDS-polyacrylamide gels (Invitrogen), transferred electrophoretically to PVDF membranes (Bio-Rad), and Western blots were performed as described.[3] Rabbit polyclonal anti-DksA antiserum was a generous gift from D. Downs (UW-Madison) and was precleared with total lysate from a strain lacking *dksA*. An HRP-conjugated secondary antibody (specific to rabbit IgG for polyclonal antibody; Santa Cruz Biotechnology) was detected using ECL+ reagent (GE Healthcare). Western blots were scanned with a Typhoon phosphorimager [GE Healthcare, 520 BP 40 Cy2, ECL+ Blue family (emission), ECL+ excitation (laser)], quantified with ImageQuant (Molecular Dynamics).

RNA extraction and primer extension for analysis of hisG promoter activity *in vivo*

Wild-type and Δ*dksA* strains 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. When cells had grown to an OD_{600} of 0.2, serine hydroxamate (0.25 mg/ml final concentration; Sigma) was added to induce amino acid starvation. After 20 min, samples were removed for immediate RNA isolation. Boiling lysis extraction of RNA and reverse transcription were performed as described.[11,38]

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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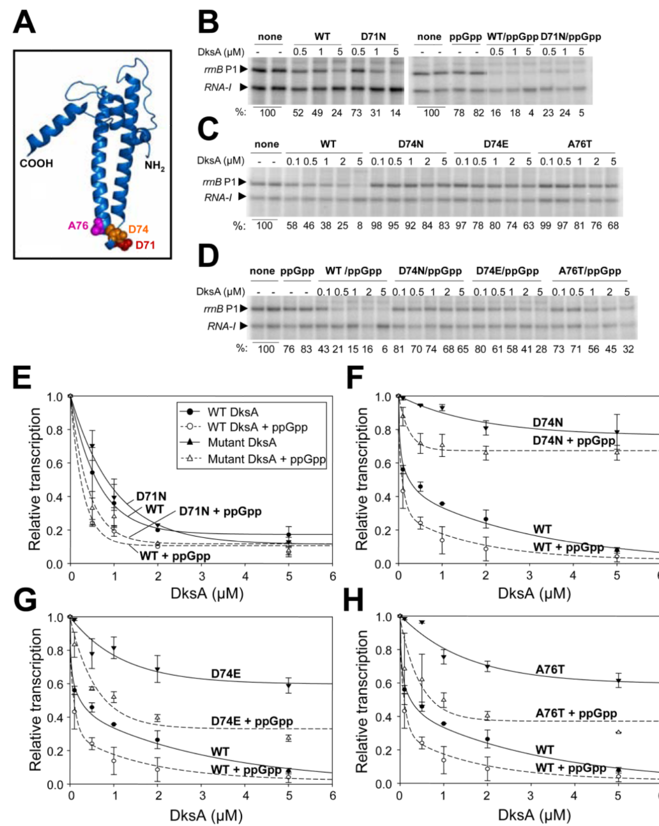

Figure 1.

DksA variants D74N, D74E, and A76T, but not D71N, are defective for inhibition of rRNA transcription *in vitro*. (A) Structure of DksA.[4] Residues D71 (red), D74 (orange), and A76 (magenta) are shown in spacefill, and the N- and C-termini are labeled. (B–D): Representative single round *in vitro* transcription experiments using plasmid templates containing the *rrnB* P1 promoter (pRLG5944; Table S1). The concentrations of wild-type or mutant DksA are indicated above each lane. ppGpp (100 μM) was included in the indicated reactions. The percentage of transcript from the *rrnB* P1 promoter relative to that without DksA or ppGpp is indicated below each lane. (E–H): *rrnB* P1 transcription ± ppGpp as a function of DksA concentration, plotted from multiple experiments like those shown in panels B–D.

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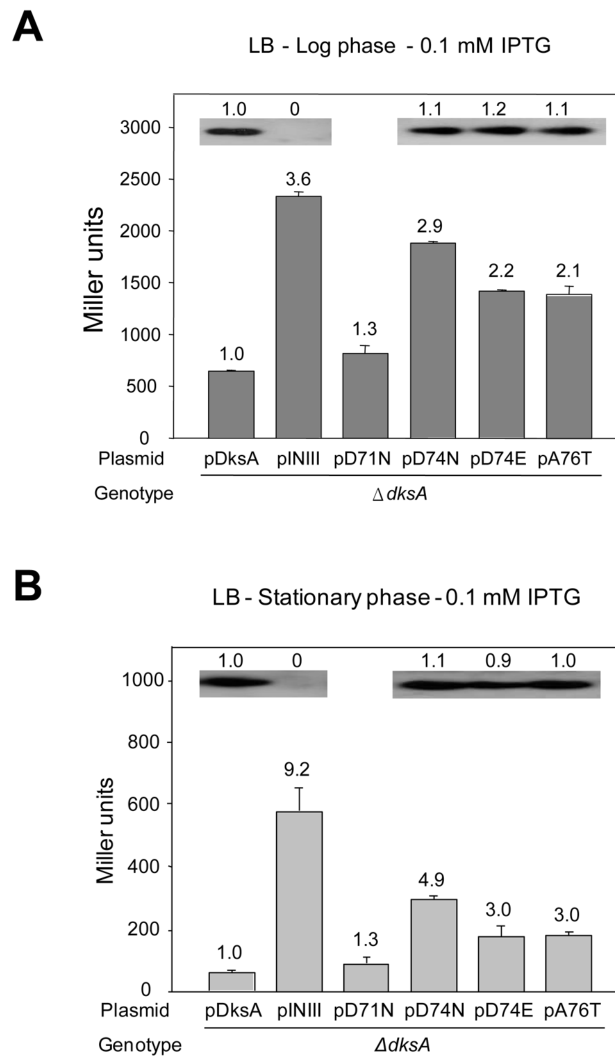

Figure 2.

DksA variants D74N, D74E, and A76T, but not D71N, are defective for inhibition of rRNA transcription *in vivo*. The bars indicate the amount of transcription from a chromosomal *rrnB* P1-*lacZ* fusion in a strain lacking the *dksA* gene and containing a plasmid encoding either wild-type DksA (pDksA), the plasmid vector only (pINIII), or the indicated variant DksAs. Cells were grown in LB containing 0.1 mM IPTG and 100 ug/ml ampicillin to (A) mid-log (OD_{600} ~0.4) or (B) stationary phase (OD_{600} ~5.0). Bars represent the mean β-galactosidase activities and standard deviations from four independent cultures. Numbers above each bar indicate the fold-increase relative to the strain containing the pDksA plasmid under the same conditions. Western blots shown at the top of each panel indicate that the DksA levels in the strains with the D74N, D74E, and A76T variants were at least as high as in the strain with wild-type DksA.

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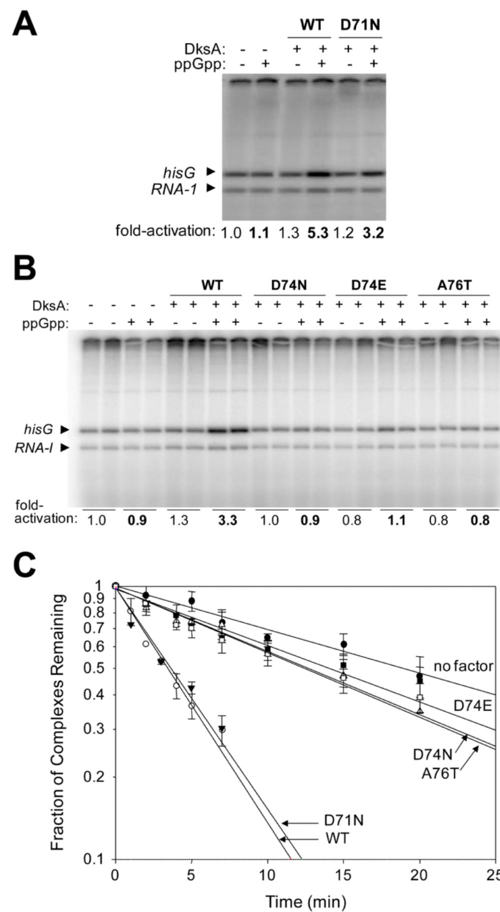

Figure 3.

DksA-D74N, D74E, and A76T, but not D71N, are defective in positive control of transcription initiation from the *hisG* promoter, and in reducing *rrnB* P1 promoter complex lifetime. Multiple-round *in vitro* transcription from the plasmid encoded P*hisG* promoter (pRLG4413) was carried out in buffer containing 165 mM NaCl ± 2 μM wild-type or mutant DksA and 100 μM ppGpp. Representative gel images are shown for (A) DksA-D71N/ppGpp and for (B) DksA-D74N, D74E, and A76T. Duplicate transcription reactions are shown in (B). Fold-activation (relative to that from reactions without DksA or ppGpp) was averaged from at least three independent experiments and is indicated below the lanes in the gel images. (C) Half-life of the RNAP-*rrnB* P1 promoter complex in the presence of 0.1 μM wild-type DksA, 0.1 μM mutant DksA or buffer only (no factor). Half-life was determined at 30°C in buffer containing 30 mM NaCl. RNAP-promoter complexes were formed on plasmid pRLG5944. The fraction of complexes remaining at the indicated times after competitor addition was determined by a transcription assay in 3 independent experiments and plotted together (see Materials and Methods).

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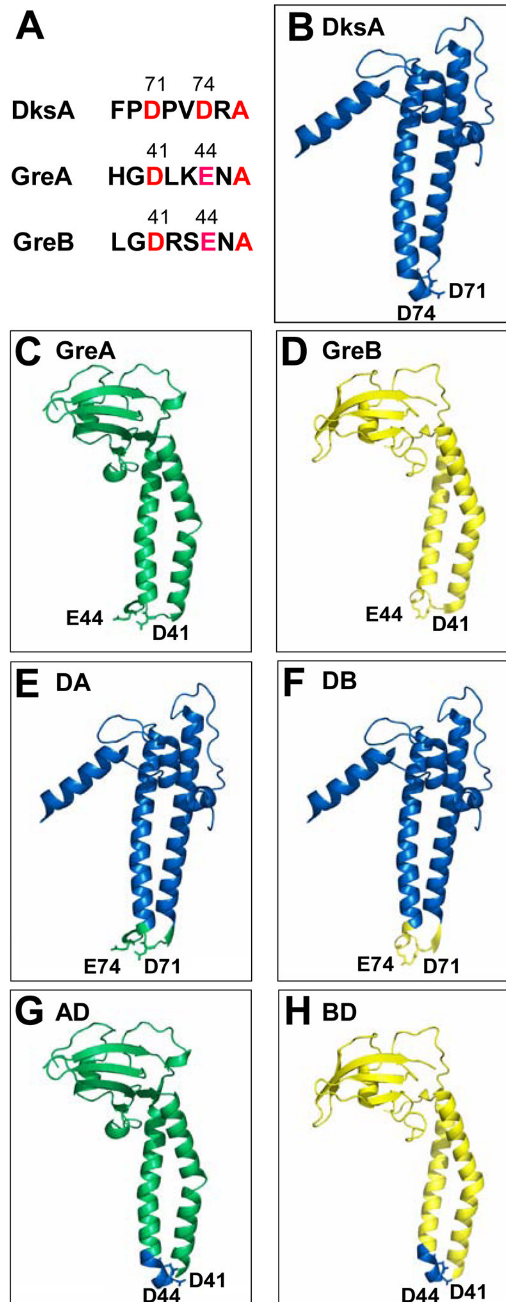

Figure 4.

DksA-Gre Chimeric Proteins. (A) Sequence alignments of the 8 amino acids in the coiled-coil tip regions of DksA and the Gre factors. The acidic tip residues (D71, D74 in DksA; D41, E44 in the Gre factors), as well as the conserved alanine residue at position 76 (DksA) or 46 (Gre factors), are in color. Models of wild-type and chimeric proteins are pictured in B–H. Regions deriving from DksA are in blue, from GreA in green, and from GreB in yellow. (B) DksA, (C) GreA, (D) GreB, (E) chimera “DA” (DksA body, GreA tip), (F) chimera “DB” (DksA body, GreB tip), (G) chimera “AD” (GreA body, DksA tip), (H) chimera “BD” (GreB body, DksA tip). Acidic tip residues are numbered according to the

body of the protein from which they were derived. The junctions of the chimeras are at residues F69 and A76 of DksA, H39 and A46 of GreA, and L39 and A46 of GreB.

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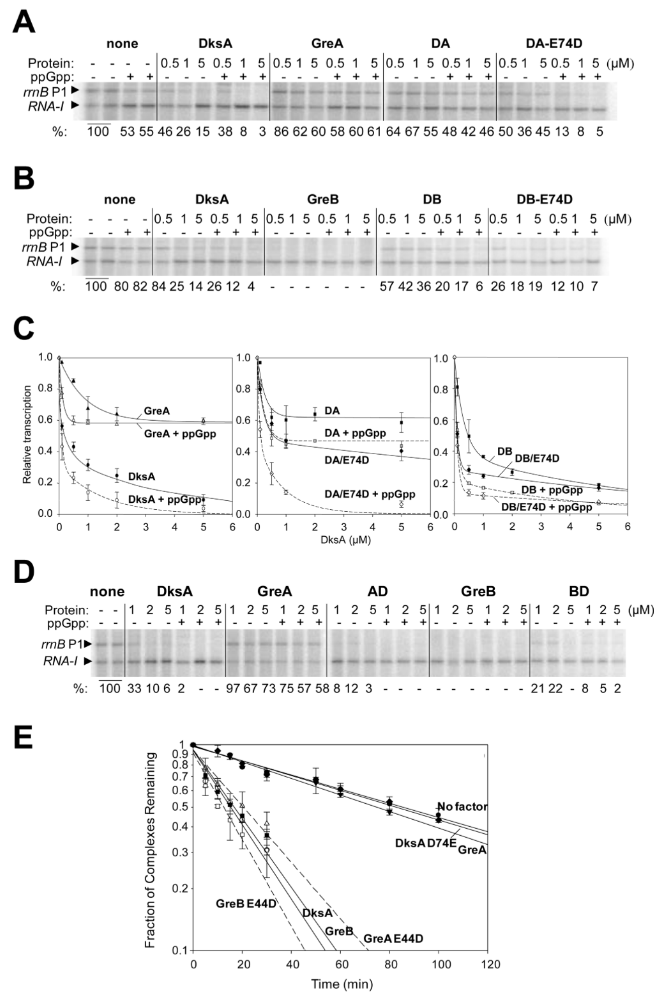

Figure 5.

An aspartate residue at position 74 in chimeric factor DA (DA-E74D), or at position 44 in GreA (E44D), confers DksA-like function. (A) Effect of chimeric factors DA or DA-E74D on inhibition of transcription from *rrnB* P1. Single round *in vitro* transcription from the *rrnB* P1 promoter was carried out with plasmid template pRLG5944 in transcription buffer containing 50 mM NaCl ± 100 μM ppGpp. (B) Effect of chimeric factors DB or DB-E74D on transcription from *rrnB* P1. (C) Plots of data from replicate transcription experiments like those shown in (A) and (B). Error bars indicate standard deviations from multiple experiments. (D) Effects of chimeric factors AD or BD on transcription from *rrnB* P1. (E) Effects of GreA-E44D or GreB-E44D on the lifetimes of *lacUV5* promoter complexes. Half-life was determined using a filter binding assay at 26°C in 100 mM KCl with 0.75 μM of each factor. The decay curves show the fraction of complexes remaining at the indicated times after heparin addition.

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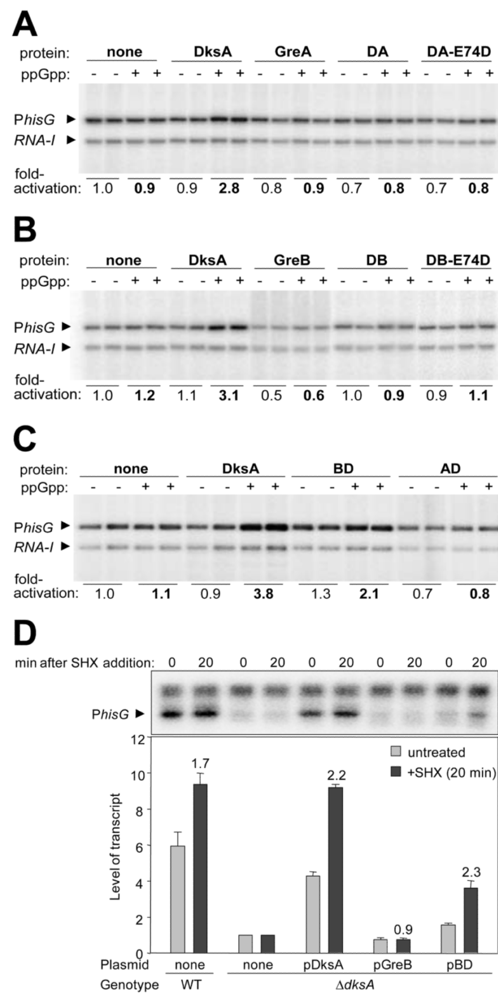

Figure 6.

Chimeric factor BD activates transcription from the *hisG* promoter *in vitro* and *in vivo*. (A–C) Multiple round *in vitro* transcription was carried out in transcription buffer containing 165 mM NaCl and plasmid template pRLG4413 ± 100 μM ppGpp and/or 2 μM DksA, Gre factor, or chimera. (A) Effect of DA or DA-E74D on transcription from P*hisG*. (B) Effect of DB or DB-E74D on transcription from P*hisG*. (C) Effect of BD or AD on transcription from P*hisG*. The BD chimera, but not the AD chimera, is able to activate transcription. (D) Effect of chimeric factor BD on promoter activity from a P*hisG* promoter-*lacZ* fusion RNA *in vivo* after amino acid starvation. DksA, GreB, or the chimeric factor BD were expressed from plasmids in strains lacking the chromosomal *dksA* gene. RNA was extracted from cells starved by addition of serine hydroxamate (SHX) or from unstarved cells and was examined by primer extension using primer 3900 (Materials and Methods and Table S2). The relevant region of the gel from a representative experiment is displayed above the graph. Numbers above each black bar indicate the fold-increase in *hisG* promoter activity in starved relative to unstarved cultures. Error bars indicate the standard deviation from four independent experiments.

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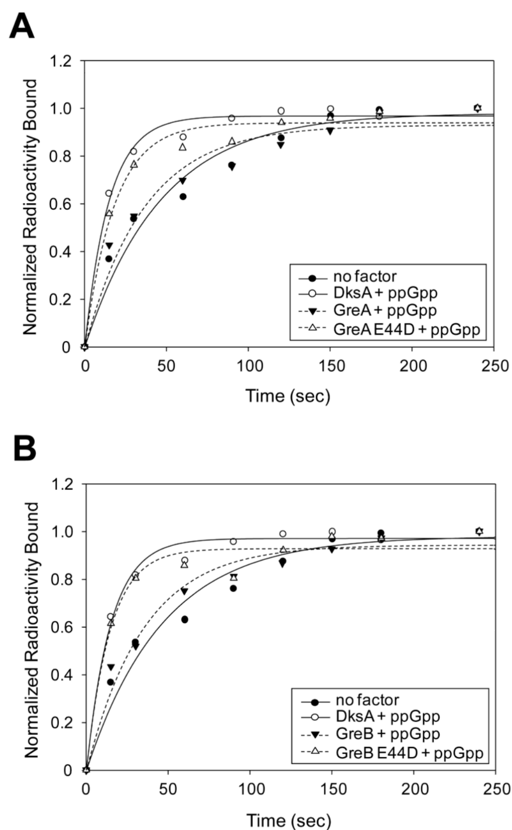

Figure 7.

An E44D substitution in GreA or GreB stimulates open complex formation. Time courses for formation of competitor resistant complexes formed by the *argI* promoter and RNAP ± DksA, GreA, GreB, GreA-E44D, or GreB-E44D using a filter binding assay. Reactions were carried out at 30°C in buffer containing 75 mM NaCl, 4 nM RNAP, 100 μM ppGpp, and 0.2 μM transcription factor. The same "no factor" and wild-type DksA curves are included in both panels for comparison with (A) GreA and GreA-E44D or (B) GreB and GreB-E44D. Plateau values for each curve are normalized to 1.0. The observed first order rate constants (k_{obs}) with absolute plateau values in parentheses were: No factor: 0.0214 s^{-1} (39%); DksA + ppGpp: 0.0673 s^{-1} (42%); GreA + ppGpp: 0.0273 s^{-1} (37%); GreA-E44D + ppGpp: 0.0553 s^{-1} (39%); GreB + ppGpp: 0.0286 s^{-1} (33%); GreB-E44D + ppGpp: 0.0690 s^{-1} (34%).

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Table 1

Activities of DksA tip mutants and chimeric DksA-Gre factors

Proteins	Binding to RNAP ($K_{d\ app}$)[a]	Percentage cleaved at max[b]	Inhibition of transcription initiation *in vitro*[c]	Stimulation of transcription initiation *in vitro*[d]
A. DksA tip mutants				
DksA	103 nM ± 14	33	+	+
DksA-D71N	129 nM ± 38	6	+	+
DksA-D74N	77 nM ± 18	7	−	−
DksA-D74E	115 nM ± 16	31	−	−
DksA-A76T	68 nM ± 9	9	−	−
B. Chimeric DksA-Gre factors				
GreA	42 nM ± 10	26	−	−
GreB	17 nM ± 2	21	+	−
DA	295 nM ± 63	34	−	−
DB	73 nM ± 11	19	+	−
DA-E74D	126 nM ± 26	11	+	−
DB-E74D	58 nM ± 7	17	+	−
AD	51 nM ± 7	13	+	−
BD	19 nM ± 4	11	+	+
GreA-E44D	39 nM ± 8	28	ND[e]	+
GreB-E44D	18 nM ± 3	20	ND[e]	+

[a]Determined by Fe^{2+}-mediated cleavage assay with RNAP core enzyme (See Ref. 25; Figs S1, S3; Materials and Methods).

[b]Maximum percentage of factor cleaved by hydroxyl radicals generated by Fe^{2+} at RNAP active center.

[c]+ indicates inhibits *rrnB* P1 transcription at least 50% as well as wild-type DksA.

[d]+ indicates stimulates P*hisG* (and other amino acid biosynthesis gene promoters) at least 50% as well as wild-type DksA.

[e]ND not done. However, factor was functional in decreasing promoter complex lifetime.