HAL
archives-ouvertes.fr

Antagonistic regulation of Escherichia coli ribosomal RNA rrnB P1 promoter activity by GreA AND DksA.

Katarzyna Potrykus, Daniel Vinella, Helen Murphy, Agnieszka Szalewska-Palasz, Richard D'Ari, Michael Cashel

▶ **To cite this version:**

Katarzyna Potrykus, Daniel Vinella, Helen Murphy, Agnieszka Szalewska-Palasz, Richard D'Ari, et al.. Antagonistic regulation of Escherichia coli ribosomal RNA rrnB P1 promoter activity by GreA AND DksA.. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2006, 281 (22), pp.15238-48. <10.1074/jbc.M601531200>. <hal-00079037>

HAL Id: hal-00079037

https://hal.archives-ouvertes.fr/hal-00079037

Submitted on 8 Jun 2006

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

This is an author-prepared PDF of a paper published in Journal of Biological Chemistry (2006) 281:15238-48

ANTAGONISTIC REGULATION OF *Escherichia coli* RIBOSOMAL RNA *rrnB* P1 PROMOTER ACTIVITY BY GreA AND DksA

Katarzyna Potrykus [‡1], Daniel Vinella [‡ §], Helen Murphy [‡], Agnieszka Szalewska-Palasz [‡ 2], Richard D'Ari [§], and Michael Cashel [‡]

From the [‡] Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland, 20892-2785, U.S.A., [§] Institut Jacques Monod, CNRS – Université Paris 6 - Université Paris 7, Paris, France.

Running title: Antagonistic regulation by GreA and DksA
Address correspondence to: Michael Cashel, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland, 20892-2785, U.S.A.; Tel.:301 4960619; FAX: 301 4960243; E-Mail: mcashel@nih.gov

The *Escherichia coli* proteins DksA, GreA and GreB are all structural homologs that bind the secondary channel of RNA polymerase (RNAP) but are thought to act at different levels of transcription. DksA, with its co-factor ppGpp, inhibits *rrnB* P1 transcription initiation while GreA and GreB activate RNAP to cleave backtracked RNA during elongational pausing. Here, *in vivo* and *in vitro* evidence reveals antagonistic regulation of *rrnB* P1 transcription initiation by Gre factors (particularly GreA) and DksA; GreA activates and DksA inhibits. DksA inhibition is epistatic to GreA activation. Both modes of regulation are ppGpp-independent *in vivo* but DksA inhibition requires ppGpp *in vitro*. Kinetic experiments and studies of *rrnB* P1-RNA polymerase complexes suggest that GreA mediates conformational changes at an initiation step in the absence of NTP substrates, even before DksA acts. GreA effects on *rrnB* P1 open complex conformation reveal a new feature of GreA distinct from its general function in elongation. Our findings support the idea that a balance of the interactions between the three secondary channel binding proteins and RNAP can provide a new mode for regulating transcription.

Structural and functional studies of bacterial and eukaryotic RNA polymerases (RNAP)[3] reveal an emerging class of transcriptional regulators: proteins that interact directly with the secondary channel - GreA, GreB, DksA, TFIIS, Microcin J25, and Gfh1 (for review see 1; 2, 3, 4). The secondary channel itself is thought to provide NTP access to a stably bound Mg^{2+} ion in the catalytic center of RNAP when template DNA and the nascent RNA-DNA hybrid occlude the main channel (5, 6). Multiple transcription factors acting at a common site on RNAP raise possibilities of new modes of transcription regulation (review, 7).

GreA and GreB are bacterial protein homologs with shared N-terminal alpha-helical coiled-coil finger structures that penetrate the secondary channel and juxtapose two acidic residues near the catalytic center; the C-terminal globular domains are implicated in RNAP binding (8, 9). Yeast RNA polymerase II transcription factor TFIIS, although structurally distinct from GreA and GreB (10), also positions a pair of conserved acidic amino acid residues near the catalytic center. In all cases, these acidic residues activate an intrinsic RNA phosphodiesterase activity of RNAP paused during elongation, leading to cleavage of nascent RNA, whose 3' end has threaded backwards relative to the catalytic center; this cleavage creates a 3’ hydroxyl near the catalytic center and restores the possibility of polymerization (11, 12, 13). Despite their structural similarities, GreA and GreB are functionally different. The length of RNA cleavage products differs and GreA, but not GreB, needs to bind before arrest in order to activate RNA cleavage (14, 15). Functions proposed for

Gre factors include: facilitating promoter escape by suppressing abortive transcription; suppressing elongational pausing or arrest; and enhancing proofreading (16; review, 1). Although blocking the passage of NTP substrates by binding of Gre factors has not been shown, channel binding by the structurally unique Microcin J25 antibacterial peptide is thought to inhibit transcription elongation directly by obstructing NTP entry (2; 17).

Transcription initiation can also be regulated through the secondary channel by DksA and (p)ppGpp, 3'-pyrophosphorylated derivatives of GDP (GTP). First, (p)ppGpp, a nearly ubiquitous nucleotide regulator of transcription in eubacteria and in plants, can be localized by co-crystallization with RNAP near the catalytic center (18). Second, DksA, despite a divergent aminoacid sequence, is a structural homolog of GreA and GreB. This allows similar modeling in the secondary channel and a proposal of nearly appropriate placement of the key pair of conserved acidic residues (19). Third, DksA and ppGpp can function both *in vitro* and *in vivo* as synergistic co-factors for negative as well as positive regulation of transcription initiation (19, 20, 21, 22). Although it requires reorientation of the pair of acidic residues, it has been proposed that DksA might anchor (p)ppGpp in one of two possible orientations near the catalytic center through Mg^{2+} ions jointly coordinated by ppGpp pyrophosphate residues, thereby stabilizing the bound co-factor and potentiating its regulatory effects (19). Whether the regulatory effect of ppGpp and DksA is positive or negative seems largely specified by the promoter discriminator sequence between the -10 and +1 region, as predicted long ago (23). DksA and ppGpp exert strong negative regulation of *rrnB* P1 and other ribosomal RNA promoters. This regulation is argued to result from enhanced destabilization and closure of RNAP - *rrnB* P1 promoter open complexes together with weakened dependence on the initiating NTP (review, 22). Positive regulation of some promoters for amino acid biosynthetic genes is attributed mainly to increasing the rate of isomerization between closed and open complexes (21).

Here, we present experiments designed to test the hypothesis that the three secondary channel binding proteins (GreA/B, DksA) can mutually compete for RNA polymerase despite known differences in the step of transcription at which they function.

EXPERIMENTAL PROCEDURES

Growth conditions, strains and plasmids: *Escherichia coli* K-12 strains used in this work (Table S1) were grown in LB broth at 37°C with appropriate antibiotic concentrations as follows: chloramphenicol (Cm) 20 μg/ml; kanamycin (Km) 40 μg/ml; tetracycline (Tc) 20 μg/ml; ampicillin (Ap) 100 μg/ml; spectinomycin (Sp) 50 μg/ml. Strains were constructed by P1*vir* transduction (24) with the following donors: CF1693 (25) as a source for Δ*spoT207*::*cat,* Δ*rel*A251::*kan*. Strains CLT254, as a source of *greA::cat*, and CLT255, as a source of *greB::kan*, were obtained from Dr. R. Weisberg.

Plasmid pBW, containing the *rrnB* P1P2 region spanning from –235 bp to +237 bp (using P1 as a reference) was constructed by removing a 370 bp *rrnB* T1T2 terminator fragment from plasmid pPS1 (26) and ligating it to an *Eco*RI and *Hind*III fragment containing the *rrnB* P1P2 region from –235 bp to +237 bp (obtained by PCR using chromosomal DNA templates). The *rrnB* P1 promoter template (-180 bp to +109 bp) was generated by PCR using pBW plasmid as template and the following primers: rrnB-180 (5'-TGCCTTTTGTATGGCAATGAC-3') and rrnB+109 (5'-AATACGCCTTCCCGCTACA-3'). The ensuing 289 bp fragment was purified using High Pure PCR Product Purification Kit (Roche Diagnostics) before use as template. We elected to use a linear template with a single promoter for all assays because inhibitory functions of DksA on *rrnB* P1 have been reported to be independent of superhelicity of the DNA template (21).

Plasmid pHM1506 was derived from pGB2 with a PSC101 origin conferring spectinomycin resistance (27) and was modified to contain *lacI*^{q} and used for expressing native *dksA* from the P*tac* promoter. For purification of C-terminal His-tagged DksA, pHM1501 was constructed by PCR cloning of the *dksA* orf from pJK537 (28) in pET21 (Novagen). The inability of the Δ*dksA* strain CF9239 to grow on glucose minimal medium is complemented by pHM1506. Plasmids used for *in vivo* experiments as well as purification of native GreA and native GreB were pDNL278 and pGF296 respectively (29).

β-galactosidase Assays - Assays for reporter activity were as described (24).

Protein Purification – His-DksA encoded by pHM1501 was purified with Ni^{2+}-NTA-agarose columns as described by Qiagen, then dialyzed against storage buffer (0.2 M Tris-Cl, pH 7.9, 0.05 M EDTA, 0.1 mM DTT, 0.1 M NaCl, 50% glycerol). GreA and GreB were purified as described in (29).

In Vitro Transcription - Transcription assays were performed in a reaction volume of 20 μl at 30°C, using 10 nM template and 30 nM RNAP *(E. coli* holoenzyme from Epicentre Technologies) in buffer containing 50 mM Tris-acetate (pH 8.0), 10 mM MgAc, 10 mM β-mercapthoethanol, 10 μg/ml bovine serum albumin, 90 mM potassium glutamate, 100 μM ATP, GTP, and CTP, and 10 μM UTP (10 μCi/reaction [α-^{32}P] UTP, Amersham), and either 250 μM GDP or 250 μM ppGpp. RNAP was pre-incubated (25^{o} C) with GDP or ppGpp for 7 min prior to the addition of potassium glutamate, and unless stated otherwise, this was followed by 7 min incubation at 30°C with DNA and the indicated GreA and/or DksA concentrations (0-600 nM). The reactions were initiated by adding NTP substrates and terminated after 10 minutes by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). In experiments varying the order of addition of GreA and DksA, the protein added first was pre-incubated with RNAP and DNA, and the second protein was added together with NTPs. In single round experiments, 100 μg/ml heparin was added. Samples were analyzed on 7 M urea, 6% polyacrylamide sequencing gels and quantified by phosphorimaging on a Molecular Dynamics imaging system.

Promoter Escape and Open Complex Stability Assays - Promoter escape and open complex stability assays were as described in (30), except that the buffer conditions, RNAP and DNA concentrations were as specified above.

DNaseI Footprinting Assay - End labeled DNA fragments were generated by PCR using ^{32}P-end-labeled *rrnB*-180 and unlabeled *rrnB*+109 primers, and purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). RNAP (0-100 nM) was pre-incubated with 500 μM GDP, or ppGpp, at room temperature for 7 min in transcription buffer, then labeled DNA template (3 nM final) was added together with potassium glutamate (90 mM final), 600 nM GreA and/or 600 nM DksA. The final reaction volume was 20 μl. Reaction mixtures were incubated at 37°C for 15 min, treated with DNaseI (7.5 ng/reaction; Promega Corporation) for 6 min, terminated by addition of EDTA (25 mM final), concentrated by vacuum evaporation, and resuspended in 20 μl loading buffer (80% formamide, 6 M urea, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol). The samples were resolved on 7 M urea, 8% polyacrylamide sequencing gels, run in parallel with sequencing reactions obtained by using unlabeled DNA fragment as a template with ^{32}P-end-labeled *rrnB*-180 primer.

$KMnO_4$ Footprinting Assay - $KMnO_4$ footprinting of the open complexes was performed as described in (30), except that the open complexes were allowed to form for 15-30 minutes before incubating under reaction conditions described above for the DNaseI footprinting. When specified, ATP and CTP were added at 100 μM each.

Electrophoretic mobility shift assays (EMSA) - These studies were performed as described in (31). When employed, ppGpp was added at 500 μM, GreA concentration was 600 nM. The template concentration was 3 nM, and RNAP concentration was varied.

RESULTS

DksA and GreA/B effects in vivo. The structural study of GreA, GreB, and DksA suggests that they might compete for their interactions with RNAP. We tested this hypothesis *in vivo*, using an *rrnB* P1::*lacZ* ribosomal promoter operon fusion (32, 33) known to be negatively regulated by DksA and ppGpp (22). Plasmids allowing IPTG-inducible P*tac* driven expression of native GreA (pDNL278), GreB (pGF296), or DksA (pHM1506) (Fig. 1, top, middle, lower rows) were introduced into strains with or without ppGpp (columns 1, 2 and 3, 4,

respectively). Reporter activities were measured during a cycle of growth in LB broth, with or without IPTG. We also introduced mutations into each series of strains eliminating potential competing proteins (columns 2, 4). Figure 1 shows differential plots of reporter activities *vs* turbidity (A600) during growth cycles. For sufficiently low values of A600, these curves are approximately linear; the slope of such a curve is the differential rate of synthesis; these values are given in Table I, expressed as Miller units (24).

Looking first at the untransformed strains in exponential phase, it can be seen (Table I) that loss of DksA increased *rrnB* P1 transcription 2.4-fold over the wild type level, whether ppGpp was present or not. This confirms that DksA is a negative regulator of *rrnB* P1 transcription. Loss of GreA, in contrast, had no effect. Figure 1 also shows that the promoter activity of the wild type strain shut-down when a critical cell density was reached (A600~2.0). No arrest was observed in the absence of DksA and arrest occurred later in the absence of ppGpp. Despite uncertainties regarding an absolute requirement for ppGpp during entry into stationary phase, this is qualitatively consistent with a requirement for both ppGpp and DksA to restrict P1 transcription late in growth in LB broth (34, 28, 20).

The same arrest was observed at high cell densities when the strains were transformed with the three plasmids, so long as the plasmids were uninduced. Major differences were observed, however, when the proteins were induced with IPTG. Some induced conditions were unexpectedly inhibitory for growth, such as inducing GreA (but not GreB) in a *dksA* mutant and inducing DksA in all strains. We therefore reduced the inducing concentration of IPTG from the standard 1 mM to 0.01 mM (panels B,D) or 0.1 mM (panels I-L). These adjustments of IPTG also yield roughly similar levels of each protein that approximate the staining intensities of the most abundant *E. coli* proteins in crude extracts (Supplement Fig. S1).

In wild type cells, increasing GreA levels activated *rrnB* P1 transcription (Fig. 1A, Table I). Simply transforming the wild type strain with p*greA* mildly elevated the specific activity 1.4x (+/- ppGpp) and IPTG induction further activated for a total of 3.4x. A *dksA* mutation in the otherwise wild type strain increased its specific activity 2.4x and transforming with p*greA* further activated 1.4x (in the absence of ppGpp), again for a total of 3.4x. In this case however, no further activation was observed after IPTG addition, but rather a slight decrease (from 250 U to 211 U).This did not result from a difference in GreA expression in the *dksA* mutant: protein staining indicated that the mutant did not induce GreA without IPTG but induced it normally when IPTG was added (Fig. S1). That equal levels of *rrnB* P1 activation occur at such different levels of GreA may mean that activation has reached a saturation limit. Indeed, this specific activity limit of 251 U was exceeded only once, 294 U, in an uninduced *dksA* mutant in the absence of ppGpp (Table I). Nevertheless, the activation of *rrnB* P1 by GreA (at its uninduced level) is potentiated by the absence of DksA.

Comparing the middle row of panels in Fig. 1 to the top row reveals that the p*greB* plasmid had little effect in the absence of inducer. Even with IPTG, activation of *rrnB* P1 promoter activity was not observed in the wild type strain (Fig. 1A,E) and was barely noticeable in the $dksA^+$ $ppGpp^0$ strain (Fig. 1G). However, inducing the p*greB* plasmid did alleviate the *rrnB* P1 arrest late in the growth cycle (Fig. 1E). Panels F and H show that the *dksA* mutant again failed to arrest, as in panels B and D.

Shortly after addition of IPTG to growing cells containing p*dksA*, reporter expression abruptly stopped in all strains (Fig. 1I-L); this arrest in *rrnB* P1 activity did not require ppGpp or GreA. Note that the ordinate scale in the last row of panels (I-L) is amplified 4x relative to upper row panels to contrast inhibition of *rrnB* P1 transcription with low activity levels. The block of *rrnB* P1 transcription when DksA was overexpressed was both more rapid and more complete than the growth limitation observed under these conditions.

These results are compatible with the hypothesis that DksA and GreA compete with each other, the latter activating *rrnB* P1 transcription, the former inhibiting it. They can be viewed as non-physiological responses because of the abnormally high level of IPTG induced proteins. If physiological function requires a balanced ratio of proteins then simultaneous induction of putative protein competitors should eliminate changes in promoter regulation. To test

this, we constructed strains with two compatible plasmids allowing IPTG induction of both DksA (pHM1506) and GreA (pDNL278). Co-induction of DksA and GreA activated *rrnB* P1 much the same as inducing GreA alone, overriding inhibitory effects of DksA alone (Supplement Fig. S2). Co-induction of GreB and DksA protected from inhibitory effects of DksA, but did not activate like GreA (Supplement Fig. S2). Evidently, balanced levels of these proteins have physiological effects regardless of their absolute concentration.

These *in vivo* experiments confirm the negative effect of DksA on *rrnB* P1 activity and suggest that excess GreA, and to a lesser extent excess GreB, can counteract it. We now ask whether these observations can be reproduced and understood in a purified transcription system.

GreA activation, not DksA inhibition, requires its presence during open complex formation. We first asked whether GreA stimulation and DksA inhibition of *rrnB* P1 transcription occurs during multiround transcription using purified GreA and DksA proteins (see Experimental Procedures). All transcription experiments employed GDP as a control for the effects of ppGpp. Initially, RNAP (30 nM) was pre-incubated with 250 μM ppGpp (or 250 μM GDP) together with increasing amounts of GreA, and the reaction was initiated by addition of 10 nM DNA template and NTP substrates. We reasoned that since GreA is implicated in binding to the RNAP secondary channel, preincubation with RNAP might increase its ability to interact with RNAP and activate. However, preincubating with even a 10-fold molar excess of GreA over RNAP did not stimulate transcription (Fig. 2A). Pre-incubating RNAP with only ppGpp (or GDP) and initiating the reaction with GreA, template and substrates also failed to reveal GreA activation (Fig. 2B). A modest activation at higher amounts of GreA was noted when the reaction was initiated with GreA and NTPs (Fig. 2C). It became clear that to achieve maximum activation (2x to 2.5x), GreA had to be present during open complex formation, i.e. in the presence of template, starting the reaction with NTPs (Fig. 2D). As observed *in vivo*, activation did not require ppGpp but was seen using a 5- to 10-fold molar excess of GreA over RNAP (Fig. 2D).

Figure 2 also shows the results of similar assays with DksA that affirm its ability to inhibit *rrnB* P1 transcription, again at a 5- to 10-fold molar excess over RNAP. Unlike *in vivo* inhibition by DksA, *in vitro* inhibition requires ppGpp (Fig. 2, A-D). Clear ppGpp-dependent inhibition was seen under all conditions tested (Fig. 2, A-D), with a somewhat stronger effect when the reactions were started with NTPs. Evidently GreA activates and DksA inhibits *rrnB* P1 transcription *in vitro*. Experiments similar to those shown in Fig. 2 were performed with GreB and slight activation was observed with or without ppGpp (data not shown), consistent with data in Fig.1.

Simultaneous vs serial addition of GreA and DksA. We next performed reactions similar to those of Fig. 2C, but asking whether GreA and DksA compete for RNAP at the *rrnB* P1 promoter. First, GreA and DksA at concentrations varying from 30 to 600 nM were presented simultaneously to a fixed concentration of 30 nM RNAP and 10 nM DNA with ppGpp or GDP, with reactions initiated by substrate addition (Fig. 3A, B). The first set of reactions showed that DksA with ppGpp could overcome stimulation by GreA even when present in significantly lower concentrations than GreA (Fig. 3A). In the absence of ppGpp, however, DksA was no longer able to overcome GreA activation even at a 20-fold molar excess of DksA over RNAP (Fig. 3B). This is consistent with a strong ppGpp co-factor requirement *in vitro* for DksA inhibition but not with the ppGpp-independent inhibition observed *in vivo* when DksA was overproduced (Fig. 1J, L). Similar results were obtained when these experiments were repeated but allowing only a single round of transcription (data not shown).

We then asked whether allowing the first protein to be present during open complex formation could be reversed by a brief exposure to the second protein. This was accomplished under conditions similar to Fig. 2D, but utilizing single round assays and initiating the reaction by the addition of the second protein together with NTP substrates (Fig. 3C-F). When GreA was added first in the presence of ppGpp, the activation by even a 20-fold molar excess of GreA over RNAP was progressively abolished by initiating the reaction with increasing amounts of DksA, NTP substrates, and heparin (Fig. 3C). Again, this reversal

required the presence of ppGpp (Fig. 3D). In contrast, even a 20-fold molar excess of GreA over RNAP did not reverse DksA inhibition in the presence of ppGpp (Fig. 3E). This was not simply because the stimulatory effect of GreA was abolished when it was added after open complexes had formed since control experiments with GDP show that GreA was able to activate *rrnB* P1 under similar conditions (Fig. 3F), although to a lesser extent than in Fig. 3B, D. Taken together, these experiments support the conclusion that, in the presence of ppGpp, transcription inhibition by DksA is dominant over *in vitro* activation of the *rrnB* P1 promoter by GreA

GreA and DksA effects on RNAP-DNA complex conformation. We next wished to document that the GreA- and/or DksA-sensitive steps in rrnB P1 transcription do indeed both occur at a stage of initiation, as implied by Fig. 3, by asking whether there are conformational changes in open complexes formed in the presence of these transcription regulators. Open binary complexes of rrnB P1 promoters and RNAP previously have been reported as too unstable for footprinting unless ternary complexes are created by a limited number of phosphodiester bonds in the presence of ATP and CTP (35). Nevertheless, we were able to obtain DNaseI footprints with RNA polymerase on double stranded DNA labeled on the 5' end of the nontemplate strand. Footprints in the absence of initiating substrates are shown in Fig. 4A using varying amounts of RNAP, 500 μM ppGpp or GDP, with or without 600 nM GreA and DksA added alone, simultaneously, or serially. We found protection with RNAP alone, with ppGpp or with GDP extending from -57 to about +30 (Fig. 4A, lanes 1-3). This protection pattern was not appreciably altered by adding DksA, whether alone, with GDP, or with ppGpp (Fig. 4A, lanes 4-6, 7-9, 19-21; Fig. 5AB). Adding GreA, especially in the presence of ppGpp (as compared to GDP) reduced DNaseI protection in the region from -35 to +30 (Fig. 4A, lanes 10-12, 22-24; Fig. 5AB). Adding DksA after preincubating GreA with RNAP and ppGpp largely eliminated the reduced protection by GreA, thereby restoring the DNaseI footprint to one characteristic of RNAP alone (Fig. 4A, lanes 2-3, 13-15, 25-27; Fig. 5AB). Adding DksA simultaneously with GreA (with ppGpp) seemed slightly less effective at reversing the GreA footprint change than adding DksA last (Fig. 4A, lanes 25-27, 28-30; Fig. 5B).

These experiments were repeated with ATP and CTP present to allow formation of initial RNA diesters and stabilization of RNAP-DNA open complexes (35) (Fig. 4B, Fig. 5CD). Protection patterns were similar except that GreA reversed protection now only in the -35 to -10 region (while protection persisted from -10 to +30) (Fig. 4B, lanes 10-12, 22-24; Fig. 5CD). Another difference is that the ppGpp specificity of GreA effects shown in Fig. 4A disappears in Fig. 4B; reversal of protection occurred in the presence of GDP as well as with ppGpp. DksA alone had no effect on the protection pattern of RNA polymerase (+/- ppGpp), but DksA in the presence of ppGpp restored the protection diminished by GreA (compare pairs of lanes 11-12, 14-15 and 17-18 with 23-24, 26-27 and 29-30 Fig. 4B; Fig. 5CD). This conclusion is in agreement with results presented in Fig. 3 where DksA was unable to overcome effects of GreA in the absence of ppGpp. The fact that GreA induces a change in the footprint in the presence of ppGpp but not GDP (Fig. 4A, compare lanes 23-24 and 11-12) may indicate an additional effect of GreA to overcome the negative effect of ppGpp and to activate transcription. To confirm the promoter specificity of the footprints, similar experiments were performed with a template bearing scrambled *rrnB* P1 -10 and -35 regions; even in the presence of ATP and CTP no DNaseI protection was obtained (data not shown). Note that GreA and DksA yielded no observable footprint in the absence of RNAP (Fig.4 AB).

We also performed $KMnO_4$ footprinting to ask whether single stranded DNA was generated by conditions similar to those presented in Fig. 4A. These footprints were found to be weak without ATP and CTP but unchanged by GreA and DksA. Parallel studies of stable complexes in the presence of ATP and CTP, similar to Fig. 4B, also did not reveal GreA- or DksA-dependent differences (data not shown). This implies that the DNaseI footprints in Fig. 4A reflect the formation of open complex intermediates and that the single stranded DNA bubbles are unchanged in the stable open complexes visualized in Fig. 4B. The effects of GreA on the extent of open complex formation seemed minimal from electrophoretic mobility

shift assays without NTP substrates (Fig. 6A). In the presence of ATP and CTP a more striking effect of GreA on the formation of complex III was found (Fig. 6B), suggesting a more lasting conformational effect (as visualized in Figs. 4B, 5CD). Complex III is judged to be the only active complex seen, as seen from its unique disappearance in the presence of all four NTP substrates (data not shown).

We were surprised that DksA did not alter the open complexes in Fig. 4A, B, although it is a strong inhibitor of *rrnB* P1 transcription. To visualize the effect of DksA better, we lowered the levels of RNAP to the point where a stable footprint was not well defined and then asked whether DksA or GreA altered the footprint. With RNAP present at 25 nM, adding 600 nM DksA (but not GreA) gave an increased level of protection in the presence of either GDP or ppGpp (Fig. 7). The protection afforded by DksA with 25 nM RNAP seems to be nearly the equivalent of 50 nM RNAP without DksA. This shows that stable open complexes are facilitated by DksA even at more dilute concentrations of RNAP (25 nM) than those in Fig.4B (50-100 nM).

DISCUSSION

Cellular experiments do not support a simple competition hypothesis. A simple prediction of the hypothesis tested here was that secondary channel interactions between GreA/B, DksA and RNAP dictate functional competition in transcription. The prediction is that changing the abundance of one protein should alter the regulatory effects of each of the others.

Measurements of effects of mutation and/or overproduction of the three proteins on transcription of the *rrnB* P1 *in vivo* are not strictly consistent with this hypothesis. First, GreB overexpression during exponential growth does not alter activity, even if DksA is abolished by mutation. In contrast, overexpression of GreA activates this promoter, depending on the abundance of DksA. Second, overexpression of DksA inhibits activity of the same promoter independent of GreA levels. Nevertheless, there exists an antagonistic regulation between GreA and DksA. A high induced level of GreA in a *dksA* mutant, but not in *dksA*$^+$, inhibits growth. The wild type level of DksA protects the cell against the potentially inhibitory effect of a high level of GreA. We believe growth inhibition by overproduction of a protein is common, but conditional inhibition depending on both overexpression of one protein and the absence of another protein is not. The cause of growth inhibition by excess GreA in the absence of DksA remains obscure.

It is puzzling that DksA induction inhibits growth even in the absence of ppGpp (Fig. 1K), because it is a co-factor of DksA for the negative regulation of rRNA synthesis (see Introduction). We also find ppGpp to be required for DksA-mediated transcription inhibition *in vitro* (Fig. 2). The explanation might relate to observations that very high (200x) molar ratios of DksA to RNAP inhibit *rrnB* P1 transcription *in vitro* even when ppGpp is absent (20). Nevertheless, the effects noted above on cell growth seem to reflect a need for a balance between the cellular content of GreA and DksA. This implies that an altered hypothesis might be that mutual competition between the three proteins is specified by unique binary combinations. Support for this possibility comes from two observations. First is that simultaneous overexpression of GreB and DksA reverses the growth toxicity of DksA without altering *rrnB* P1 activity (Supplement Fig. S2). Second, overexpression of both GreA and DksA reverses inhibition of *rrnB* P1 activity (Supplement Fig. S2).

The balance between these three proteins can also affect the arrest of *rrnB* P1 activity that normally occurs at A600 ~2 (Fig. 1A). This arrest is bypassed in the absence of DksA (Fig. 1B) or when high levels of GreA are induced in a wild type strain (Fig. 1A). It seems that the normal level of DksA prevents the normal level of GreA from activating *rrnB* P1 in stationary phase, just as it prevents GreA-mediated growth inhibition during exponential growth. GreB, when overexpressed, shares with GreA the ability to bypass the arrest of *rrnB* P1 transcription (Fig. 1E). Arrest of *rrnB* P1 transcription in wild type cells probably reflects entry into early stationary phase, since it requires both DksA and ppGpp (see results). Arrest of *rrnB* P1 activity is promoter specific because no arrest was observed with a *lacUV5* promoter operon fusion in *greA*, *greB*, or *dksA* strains (data not shown).

We believe this is the first evidence that the balance of the three secondary channel binding proteins has functional significance and raises new possibilities for transcription regulation.

Similarities and differences in vivo and in vitro, for GreA and DksA effects. A direct activating effect of GreA on transcription has been found. Maximal activation requires its presence during open complex formation. Multiple round transcription reactions reproduced the activation of *rrnB* P1 transcription by GreA to nearly the same extent (2.5x) as *in vivo* (3.4x); the ppGpp independence of this effect was also verified (Fig. 2).

Several parameters associated with initiation were not altered by GreA. The presence of GreA (or DksA) had no effect on the low level of abortive transcripts under all conditions of Fig. 2A-D (data not shown). Also, measuring promoter escape by back extrapolating closely timed kinetic samples failed to reveal significant differences in the presence of GreA (data not shown). Finally, open complex stabilities, determined with a heparin challenge, were unaffected by the presence of GreA (data not shown).

DksA added in parallel *in vitro* experiments allowed us to verify published observations of a co-factor requirement for ppGpp to inhibit *rrnB* P1 transcription (20). Mixtures of GreA and DksA presented simultaneously to RNAP at varying ratios to each other and to RNAP revealed that even brief exposures to DksA could inhibit and prevent or reverse GreA activation (Fig.3). Like DksA-mediated inhibition, this reversal of GreA activation was ppGpp-dependent.

DksA and GreA alter RNAP-DNA complex conformation. DNaseI footprints of RNAP with GreA and/or DksA and the *rrnB* P1 promoter DNA revealed the existence of a specific *rrnB* P1 promoter binary open complex without the need for nucleotide substrates or accessory proteins (Fig. 4A). This footprint persisted unchanged in the presence high DksA levels, with or without ppGpp, as if DksA did not destabilize the open complex. When GreA was substituted for DksA, the protection pattern was diminished, especially in the presence of ppGpp. The GreA-mediated change in DNaseI protection pattern was partially or completely reversed by DksA addition in the presence of ppGpp but not GDP. A change in the intensity of a footprint, without altering the extent of the protected region, could result from a change in RNAP promoter affinity. However, this possibility was ruled out by EMSA, where we did not observe decreased binding of RNAP to *rrnB* P1 region (Fig. 6). Instead we found that GreA even enhanced the formation of active RNAP-DNA complexes in the presence of ATP and CTP (Fig. 6B). We therefore interpret the GreA-induced change in the DNaseI footprint (Fig. 4A; Fig. 5AB) to represent a conformational change in the RNAP-promoter binary complex.

Furthermore, the footprints imply that GreA acts before DksA during initiation, in the absence of NTPs, which is consistent with the order of addition experiments (Fig. 3C-F). Analogous footprints obtained in the presence of ATP and CTP were similar except that the footprint pattern with GreA was reversed at slightly different sequence positions by DksA. With ATP and CTP, GreA reversal now occurred in the presence of ppGpp and to a lesser extent in the presence of GDP (Fig. 4B; Fig. 5CD). Here, we are more confident of a conformational change because the extent of the footprint was changed by GreA, not just its intensity. This footprint covered only about 30 bp of DNA, which corresponds to expectations for an elongating complex (36). This difference is interesting, because without GreA present one would expect the first phosphodiester bond to be formed, and yet the footprint has an extent similar to an open binary complex. Perhaps, GreA by binding to RNAP in the presence of the two initiating nucleotides is able to tilt the balance from an open initiating complex toward an elongating complex. Alternatively, DksA inhibition may prevail despite a 20-fold molar excess of GreA (Fig. 3) because of a higher affinity of DksA for RNA polymerase than GreA as appears to be the case from Fig. 7.

Apparently, GreA and DksA can exist in distinct complexes with RNAP and the *rrnB* P1 promoter. As stated above, it seems that GreA can modify the conformation of RNAP complexes with DNA. It is important to note that the GreA effects we see on open complex conformation in the absence of NTP substrates are very different from previous reports that in the presence of NTP substrates GreA either stimulates the initiation

process after phosphodiester bond formation has begun (16, 37) or prevents the formation of inactive moribund complexes at the lambda pR promoter (38).

DksA does not seem to change the conformation of the open complex, but when present with GreA, it restores a conformation that mimics what is seen when GreA is absent. It is interesting in this respect that DksA alone appears to decrease the amount of RNAP necessary to obtain a DNaseI footprint corresponding to an open complex (Fig. 7). This could mean that DksA facilitates DNA binding by RNAP, which could in turn occur by increasing the DNA binding constant (K_B), or by increasing the transition between closed and open complex or by stabilizing the open complex. Two different approaches for estimating conformational change (EMSA and footprints), indicate that DksA does not cause the collapse of open complexes under our conditions. These observations conflict with existing views that DksA and ppGpp are thought to increase the instability of *rrnB* P1 open complexes and simultaneously weaken the binding for initiating nucleotides (20). An analogous finding to ours was reported by Maitra *et al.* (31), for a *rpoC*Δ(312-314) mutant that suppresses ppGpp deficiency. Interpretations by Paul *et al.* (20) as to the instability of open complexes based upon the heparin chase experiments were challenged by the discovery of stable, but inactive promoter-RNAP binary complexes formed by this *rpoC* mutant.

Considerations of GreA and DksA actions. The conclusion that GreA participates in initiation goes against existing information on its mode of action (Fig. 8). However there is evidence that GreA, unlike GreB, must be added before pausing actually occurs in order to activate cleavage of backtracked RNA (14). Perhaps there is a GreA binding site that is inaccessible in paused RNAP but accessible during initiation. The new finding here is that GreA can be bound during open complex formation (Fig. 8). It could even be imagined that GreA joins RNAP during initiation and is carried along during elongation to later perform its well established functions. This does not necessarily exclude the binding of GreA during elongation. Speculations about assembly of a GreA-containing transcription complex during initiation are reminiscent of eukaryotic transcription. Although the eukaryotic GreA homolog, TFIIS, is thought to bind RNAP during elongation, there is a report that it may play a role during initiation with Gal4-dependent promoters (39).

If GreA assembles in such a complex, then either GreA binding does not obstruct the secondary channel or NTP entry can occur by an alternative route. Only GreB has been co-crystallized with RNAP (9). Modeling of the docking of both GreA and DksA structures (1, 19) revealed that the key acidic residues of DksA require rotation to achieve the same orientation as in GreA and GreB. We wonder whether this orientation might determine activation for GreA or GreB and inhibition for DksA. Similar speculations have been raised by Lamour *et al.* (3) and Symersky *et al.* (4), in their work on Gfh1, a *Thermus thermophilus* Gre-like homolog responsible for transcription inhibition. Compared to GreA, there are four acidic residues in the Gfh1 finger domain, which are again in a different orientation. The authors hypothesize that these residues impair transcription by chelating Mg^{2+} in the RNAP active site. Our finding that GreA activates transcription initiation suggests that additional studies are required before proposing a more detailed mechanism for GreA.

If indeed GreA functions in initiation as argued here, it may be possible to obtain GreA mutants that differentially affect initiation but not elongation, or *vice versa*. GreA effects on open complex conformation before RNA diesters are formed might suggest that cleavage or the conserved acidic residues of GreA might not be activation requirements. Since GreA seems to act at the stage of transcription initiation, DksA in turn may exert unexpected additional effect on elongation. Although preliminary studies *in vitro* (19) seem to discourage this idea, a similar investigation *in vivo* may prove interesting.

Finally, it was reported recently by Trautinger *et al.* (40) that deleting *dksA* sensitizes cells to mitomycin C. Intriguingly, introducing a *greA* deletion in addition to *dksA* reversed this phenotype, consistent with our proposal that GreA and DksA counteract each other's actions. One of the most important implications of our finding is that cellular regulation of transcription can be achieved by a balance of antagonistic activities of

RNA polymerase secondary channel binding factors: DksA, GreA, and to a lesser degree GreB.

While in the process of revising our manuscript, a publication by Susa *et al.* (41) appeared describing genes whose transcription was impaired by disrupting GreA and GreB. The mechanism explaining these effects is argued to be reversal of formation of abortive moribund complexes. It will be interesting to see if this process is related to the phenomenon we observed.

ACKNOWLEDGEMENTS

We thank Robert Weisberg for providing strains CLT254 and CLT255. We thank Jim Hernandez for plasmids (pDNL278, pGF296), for help with the EMSA experiments, and for insightful discussions. We are grateful to Lilian Hsu for the protocol for purifying GreA and GreB. Rajendran Harinarayanan's and Bill Whalen's comments on the manuscript are appreciated. Constructive criticism from the Friday Seminar group is gratefully acknowledged. Martin Blum facilitated our *in vivo* experiments. This research was supported in part by the Intramural Research Program of the NIH, NICHD.

REFERENCES

1. Borukhov, S., Lee, J., and Laptenko, O. (2005) *Mol. Microbiol.* **55:** 1315-1324
2. Mukhopadhyay, J., Sineva, E., Knight, J., Levy, R.M., and Ebright, R.H. (2004) *Mol. Cell* **14:** 739-751
3. Lamour, V., Hogan, B. P., Erie, D. A., and Darst, S. E. (2006) *J. Mol. Biol.* **356:** 179-188
4. Symersky, J., Perederina, A., Vassylyeva, M. N., Svetlov, V., Artsimovitch, I., and Vassylyev, D. G. (2006) *J. Biol. Chem.* **281:** 1309-1312
5. Zhang, G., Campbell, E.A. Minakhin, L., Richter, C., Severinov, K., and Darst, S.A. (1999) *Cell* **98:** 811-824
6. Vassylyev, D.G., Sekine, S., Laptenko, O., Lee, J., Vassylyeva, M.N., Borukhov, S., and Yokoyama, S. (2002) *Nature* **417:** 712-719
7. Nickels, B. E., and Hochschild, A. (2004) *Cell* **118:** 281-284
8. Stebbins, C.E., Borukhov, S., Orlova, M., Polyakov, M., Goldfarb, A., and Darst, S.A. (1995) *Nature* **373:** 636-640
9. Opalka, N., Chlenov, M., Chacon, P., Rice, W.J., Wriggers, W., and Darst, S.A. (2003) *Cell* **114:** 335-345
10. Kettenberger, H., Armache, K.-J., and Cramer, P. (2003) *Cell* **114:** 347-357
11. Komissarova, N., and Kashlev, M. (1997) *Proc. Natl. Acad. Sci. USA* **94:** 1755-1780
12. Nudler, E., Mustaev,A., Lukhtanov,E., and Goldfarb, A. (1997) *Cell* **89:** 33-41
13. Sosunova, E., Sosunov, V., Kozlov, M., Nikiforov, V., Golfarb, A., and Mustaev, A. (2003) *Proc. Natl. Acad. Sci. USA* **100:** 15469-15474
14. Borukhov, S., Sagitov, V., and Goldfarb, A. (1993) *Cell* **72:** 459-466
15. Laptenko, O., Lee, J., Lomakin, I., and Borukhov, S. (2003) *EMBO J.* **22:** 6322-6334
16. Hsu, L.M., Vo, N.V., and Chamberlin, M.J. (1995) *Proc. Natl. Acad. Sci. USA.* **92:** 11588-11592
17. Adelman, K., Yuzenkova, J., La Porta, A., Zenkin, N., Lee, J, Lis, J.T., Borukhov, S. Wang, M.D., and Severinov, K. (2004) *Mol. Cell* **14:** 753-762
18. Artsimovitch, I., Patlan, V., Sekine, S., Vassylyeva, M.N., Hosaka, T., Ochi, K., Yokoyama, S., and Vassylyev, D. (2004) *Cell* **117:** 299-310
19. Perederina, A., Svetlov, V., Vassylyeva, M. N., Tahirov, T. H., Yokoyama, S., Artsimovitch, I., and Vassylyev, D. G. (2004) *Cell* **118:** 297-309
20. Paul, B. J., Barker, M. M., Ross, W., Schneider, D. A., Webb, C., Foster, J.W., and Gourse, R. L. (2004) *Cell* **118:** 311-322
21. Paul, B. J., Berkmen, M. B., and Gourse, R. L. (2005) *Proc. Natl. Acad. Sci. USA* **102:** 7823-7828
22. Paul, B. J., Ross, W., Gaal, T., and Gourse, R. L., (2004)*Annu. Rev. Genet.* **38:** 749-770

23. Travers, A. A. (1980) *J. Bacteriol.* **141:** 973-976
24. Miller, J.H. (1972) *A short course in Bacterial Genetics.* Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York, USA
25. Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1991) *J. Biol. Chem.* **266:** 5980-5990
26. Sarmientos, P., Sylvester, J. E., Contente, S., and Cashel, M. (1983) *Cell* **32:** 1337-1346
27. Churchward, G., Belin, D., and Nagamine, Y. (1984) *Gene* **31:** 165-171
28. Brown, L., Gentry, D., Elliott, T., and Cashel, M. (2002) *J. Bacteriol.* **184:** 4455-4465
29. Feng, G.H., Lee, D.N., Wang, D., Chan, C., and Landick, R. (1994) *J. Biol. Chem.* **269:** 22282-22294
30. Potrykus, K., Wegrzyn, G., and Hernandez, V.J., (2004) *J. Biol. Chem.* **279:** 19860-19866
31. Maitra, A., Shulgina, I., and Hernandez, V.J. (2005) *Mol. Cell* **17:** 817-829
32. Hernandez, V.J. and Bremer, H. (1990) *J Biol. Chem.* **265:** 11605-11614
33. Vinella, D., Cashel, M., and D'Ari, R. (2000) *Genetics* **156:** 1483-1492
34. Aviv, M., Giladi, H., Oppenheim, A. B., and Glaser, G. (1996) *FEMS Microbiol Lett* **140:** 7 l-76
35. Gourse, R.L. (1988) *Nucleic Acids Res.* **16:** 9789-9809
36. Krummel, B., and Chamberlin, M. J. (1992) *J. Mol. Biol.* **225:** 239-250
37. Borukhov, S., Sagitov, V., Josaitis, C.A., Gourse, R.L., and Goldfarb, A. (1993) *J. Biol. Chem.* **268:** 23477-23482
38. Sen, R., Nagai, H., and Shimamoto, N. (2001) *Genes Cells* **6:** 389-401
39. Prather, D. M., Larschan, E., and Winston, F. (2005) *Mol Cell Biol.* **25:** 2650-2659
40. Trautinger, B.W., Jaktaji, R.P., Rusakova,E., and Lloyd, R.G. (2005) *Mol. Cell* **19:** 247-25
41. Susa, M., Kubori, T., and Shimamoto, N. (2006) *Mol Microbiol* **59:** 1807-1817

FOOTNOTES

[1] On leave from the Department of Molecular Biology, University of Gdansk, Kladki 24, 80-822, Gdansk, Poland

[2] Current address: Department of Molecular Biology, University of Gdansk, Kladki 24, 80-822, Gdansk, Poland

[3] The abbreviations used are: RNAP, RNA polymerase; ppGpp, guanosine-3,5-(bis)pyrophosphate; EMSA, electrophoretic mobility shift assay.

Figure Legends

Fig.1. Overproduction of GreA, GreB, or DksA alters *rrnB* P1::*lacZ* activity. Using Δ*lacZ* strains carrying the *rrnB* P1::*lacZ* transcriptional fusion, we evaluated the effects of overproduction of GreA (panels A to D, strains transformed with pDNL278 = p*greA*), GreB (panels E to H, strains transformed with pGF296 = p*greB*), or DksA (panels I to L, strains transformed with pHM1506 = p*dksA*) on *rrnB* P1::*lacZ* activity. All strains were grown at 37°C; IPTG was added when the culture reached an OD600 of 0.10- 0.15; β-galactosidase activity was monitored periodically. Squares: untransformed strains cultivated in LB. Filled diamonds: transformed strains cultivated in LB supplemented with ampicillin (panels A to H) or with spectinomycin (panels I to L). Circles: transformed strains cultivated in the same media supplemented with IPTG at 10^{-3} M (panels A, C and E to H), 10^{-4} M (panels I to L) or 10^{-5} M (panels B and D). Although lines showing arrest of activity for A600 ≥ 2.0 seem defined by a single point in panels A,E,I and J, the lines as drawn actually depend on more extensive data not shown. The Δ*relA spoT*$^+$ strains are termed wild type here; equivalent results were obtained with *relA*$^+$ *spoT*$^+$ strains (data not shown).

Fig. 2. Effects of GreA and DksA on *in vitro* transcription initiating from the *rrnB* P1 promoter depends on the order of addition. RNAP (30 nM) was pre-incubated with either 250 μM ppGpp (or GDP), and then: (A) pre-incubated with increasing concentrations of GreA or DksA (0-300 nM) and started by the addition of DNA (10 nM) and NTP substrates; (B) initiated by the simultaneous addition of DNA, NTPs, and GreA or DksA; (C) pre-incubated with DNA to allow for open complexes to form, and then initiated by the addition of NTPs together with GreA or DksA; (D) pre-incubated with both DNA and GreA or DksA, and then initiated by the addition of NTPs. Additional controls for all panels lacking GDP or ppGpp were similar to those with GDP. The standard deviation for all values was <10%.

Fig. 3. GreA and DksA effects on *rrnB* P1 when added together. Transcription assays were performed as described in Fig. 2D, except that: (A) and (B) - increasing GreA concentrations (0-600 nM) were added simultaneously with DksA (0-600 nM) and NTPs as in 2C; reactions for panels C-F were single round under the conditions of Fig. 2D: (C) and (D) – GreA was added to the RNAP/DNA mix and the reaction was initiated by addition of NTPs with DksA; (E) and (F)- DksA was added first and the reaction was initiated by the addition of NTPs with GreA. In panels (A), (C), and (E) assays were carried out in the presence of ppGpp; in panels (B), (D), and (F) GDP was substituted.

Fig. 4. Footprinting analysis of RNAP protection at *rrnB* P1 in the presence of GreA and/or DksA. Reactions contained 3 nM double stranded template with the 3' end of the nontemplate strand labeled, 500 μM ppGpp or GDP, and GreA and/or DksA. Amounts of RNAP present were 0, 50, or 100 nM The concentrations of the RNAP used are indicated at the top of the gel. Unlabeled lanes 1-3 = neither GreA, DksA, GDP, nor ppGpp present; lanes labeled 600 nM D - only DksA was present; lanes labeled 600 nM A - only GreA was present; A→ D - samples were pre-incubated with GreA and then DksA was added; (A + D) – both proteins were added simultaneously. Lanes labeled G,A,C, or T are sequencing ladders. Numbers at the right represent nucleotide positions relative to the start site. Panel A footprints were performed in the absence of NTP substrates while Panel B footprints were in the presence of ATP and CTP. This autoradiograph is lightly exposed to better visualize the differences between the footprints.

Fig. 5. Quantitative scans of the individual lanes of the DNaseI I footprints. Selected lanes from Fig. 4 were quantitated by scanning densitometry. (A) Lanes with GDP from panel A of Fig. 4: RNAP with DksA alone (orange; lane 9), with GreA alone (red; lane 12), with GreA added first then DksA (yellow green; lane 15) and GreA together with DksA (green; lane 18). (B) Corresponding colors have been applied to lanes with ppGpp from Fig.4A (lanes 21, 24, 27 and 30). (C) A similar presentation applies to

analogous GDP control lanes from Fig.4B (lanes 8, 11, 14 and 17) as well as for (D) Footprint lanes with ppGpp from Fig.4B (20, 23, 26 and 29).

Fig. 6. Electrophoretic mobility shift assays. (A) Assays were performed with RNAP concentrations as shown: filled symbols- in the presence of ppGpp; open symbols- absence of ppGpp; RNAP alone – circles; RNAP with GreA- triangles. (B) As in panel A, but the reactions were carried out in the presence of the two initiating nucleotides, ATP and CTP. Quantitation is presented for the bands corresponding to the three complexes detected in each panel as well as for the amount of unbound DNA is presented.

Fig. 7. DksA enhances RNAP binding to the *rrnB*P1 promoter region. (A) DNaseI footprinting was performed in the absence of ppGpp at varying RNAP concentrations as indicated at the top of the gel. (B) The assay was carried out at 25 nM RNAP, but in the presence of either 500 μM ppGpp or GDP; lanes labeled A - 600 nM GreA; lanes labeled D - 600 nM DksA; lanes labeled A+D - both GreA and DksA added. The '-' label denotes conditions where RNAP alone was employed. In all cases, the initiating nucleotides ATP and CTP were present to allow stable complex formation. Sequencing ladders are indicated. Numbers at the right represent nucleotide positions relative to the start site. The boxed regions of three lanes direct attention to regions allowing comparison of DksA protective effects at 25 nM RNAP concentrations. (C) Quantitative scans of the footprint shown in panel B with or without ppGpp, corresponding to lanes with RNAP alone (red), with GreA (yellow green), with DksA (orange) and with GreA and DksA together (green).

Fig. 8. GreA acts at elongation as well as at initiation of transcription.
(A) Generally accepted view that GreA acts at the stage of transcriptional elongation. (B) During initaiton GreA binds to RNAP and activates transcription (in a ppGpp-independent manner) at the *rrnB* P1 promoter, at the stage of transcriptional complex assembly. During elongation, GreA may be carried along in the transcription complex or, if needed, a new GreA molecule may bind and rescue paused or arrested complexes, as in panel A. At the initiation stage DksA is able to overcome GreA's effect and inhibit transcription in a ppGpp-dependent manner.

Table I. Data tabulated are differential rates of synthesis of β-galactosidase expressed in Miller units (Miller, 1972). These specific activities were calculated for the growth curve regions where absolute activities increase linearly with turbidity (Fig. 1). In parentheses are the relative rates, normalized to the wild type rate.

Table I

Genotype **Plasmid**	***relA***	***greA relA***	***dksA relA***	***relA spoT***	***greA relA spoT***	***dksA relA spoT***
None	74 (≡1 x)	76 (1 x)	174 (2.4x)	92 (1.2x)	102 (1.4x)	173 (2.3x)
p*greA*	105 (1.4x)	-	250 (3.4x)	111 (1.5x)	-	294 (4.0x)
" + IPTG	251 (3.4x)	-	211 (2.9x)	242 (3.3x)	-	249 (3.4x)
p*greB*	67 (0.9x)	-	129 (1.7x)	94 (1.3x)	-	154 (2.1x)
" + IPTG	111 (1.5x)	-	134 - 183 (1.8x - 2.5x)	133 (1.8x)	-	161 - 213 (2.2x -2.9x)
p*dksA*	50 (0.7x)	59 (0.8x)	101 (1.4x)	78 (1.1)	101 (1.4x)	-
" + IPTG	< 5 (< 0.07x)	< 5 (< 0.07x)	< 5 (< 0.07x)	< 5 (< 0.07x)	< 5 (< 0.07x)	-

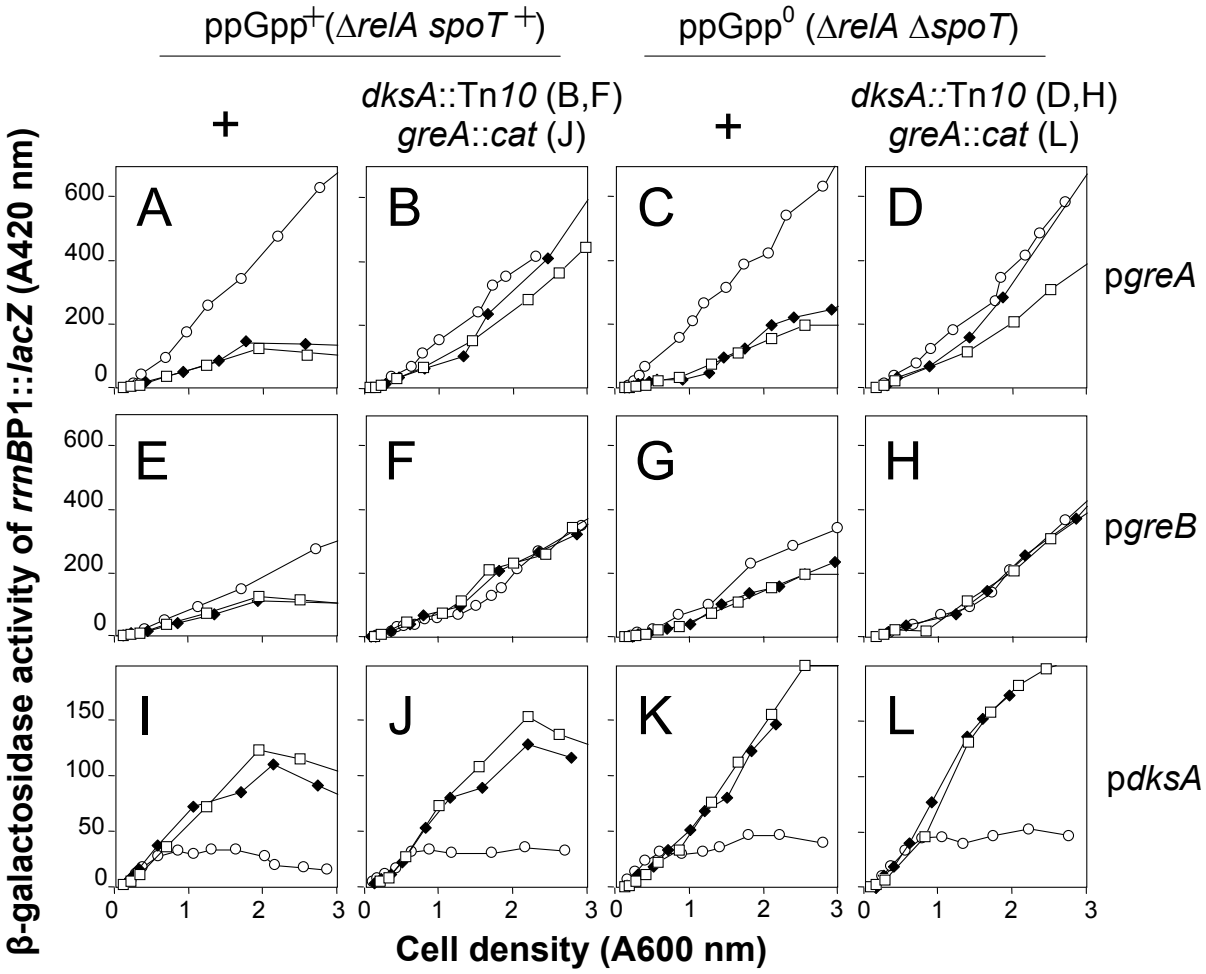

ppGpp+(ΔrelA spoT +)
ppGpp0 (ΔrelA ΔspoT)
+
dksA::Tn10 (B,F)
greA::cat (J)
+
dksA::Tn10 (D,H)
greA::cat (L)
A
B
C
D
E
F
G
H
I
J
K
L
pgreA
pgreB
pdksA
β-galactosidase activity of rrnBP1::lacZ (A420 nm)
Cell density (A600 nm)

Fig.1.

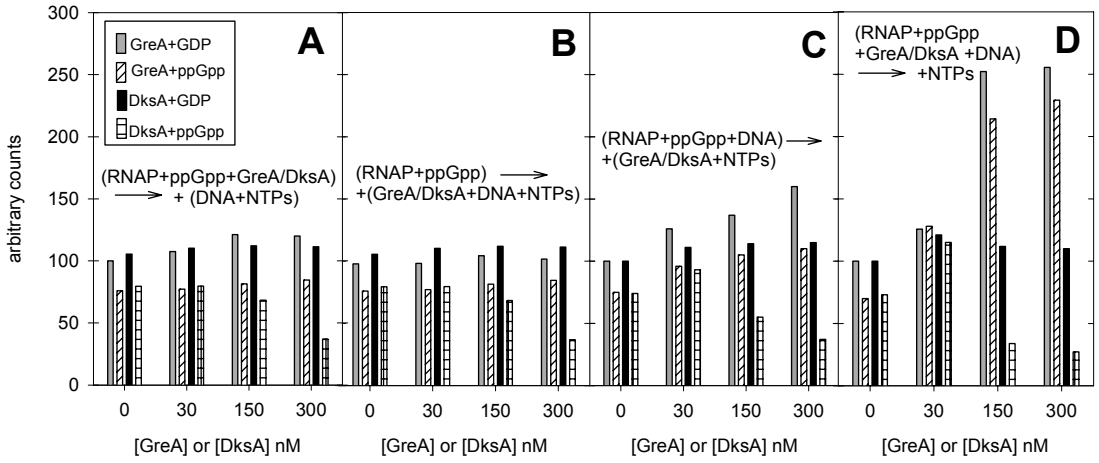

GreA+GDP
GreA+ppGpp
DksA+GDP
DksA+ppGpp
A
B
C
D
(RNAP+ppGpp+GreA/DksA) → + (DNA+NTPs)
(RNAP+ppGpp) → +(GreA/DksA+DNA+NTPs)
(RNAP+ppGpp+DNA) → +(GreA/DksA+NTPs)
(RNAP+ppGpp +GreA/DksA +DNA) → +NTPs
arbitrary counts
0
50
100
150
200
250
300
0
30
150
300
[GreA] or [DksA] nM

Fig.2.

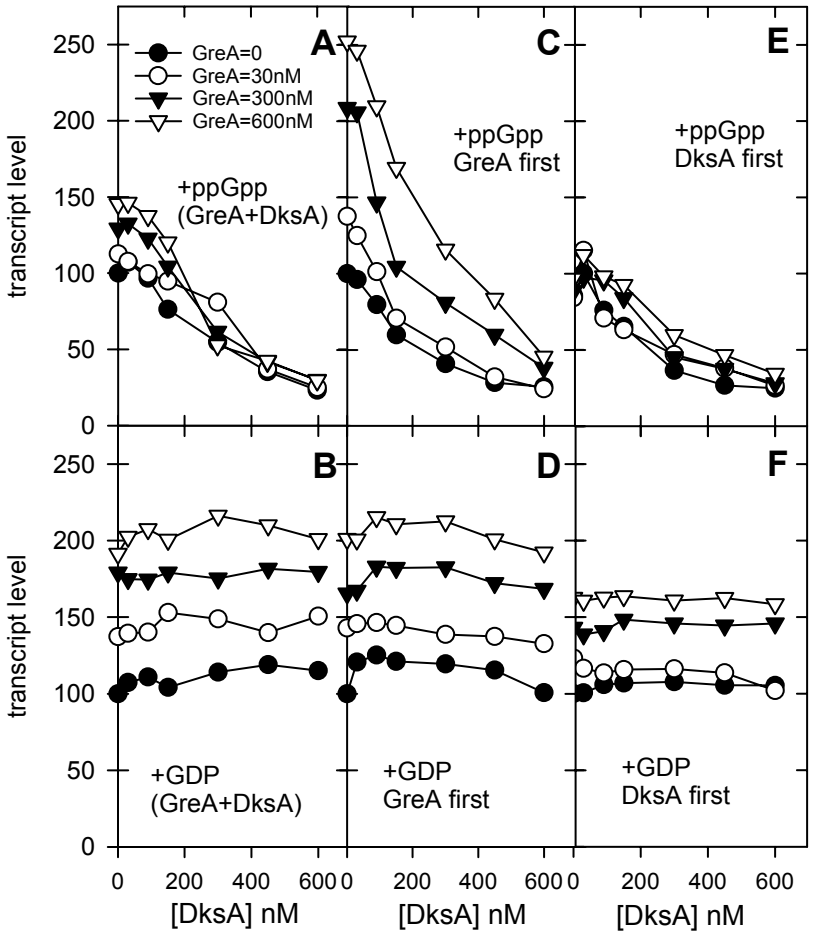

A
B
C
D
E
F
GreA=0
GreA=30nM
GreA=300nM
GreA=600nM
+ppGpp
(GreA+DksA)
+ppGpp
GreA first
+ppGpp
DksA first
+GDP
(GreA+DksA)
+GDP
GreA first
+GDP
DksA first
transcript level
transcript level
[DksA] nM
[DksA] nM
[DksA] nM
0
50
100
150
200
250
0
200
400
600

Fig.3.

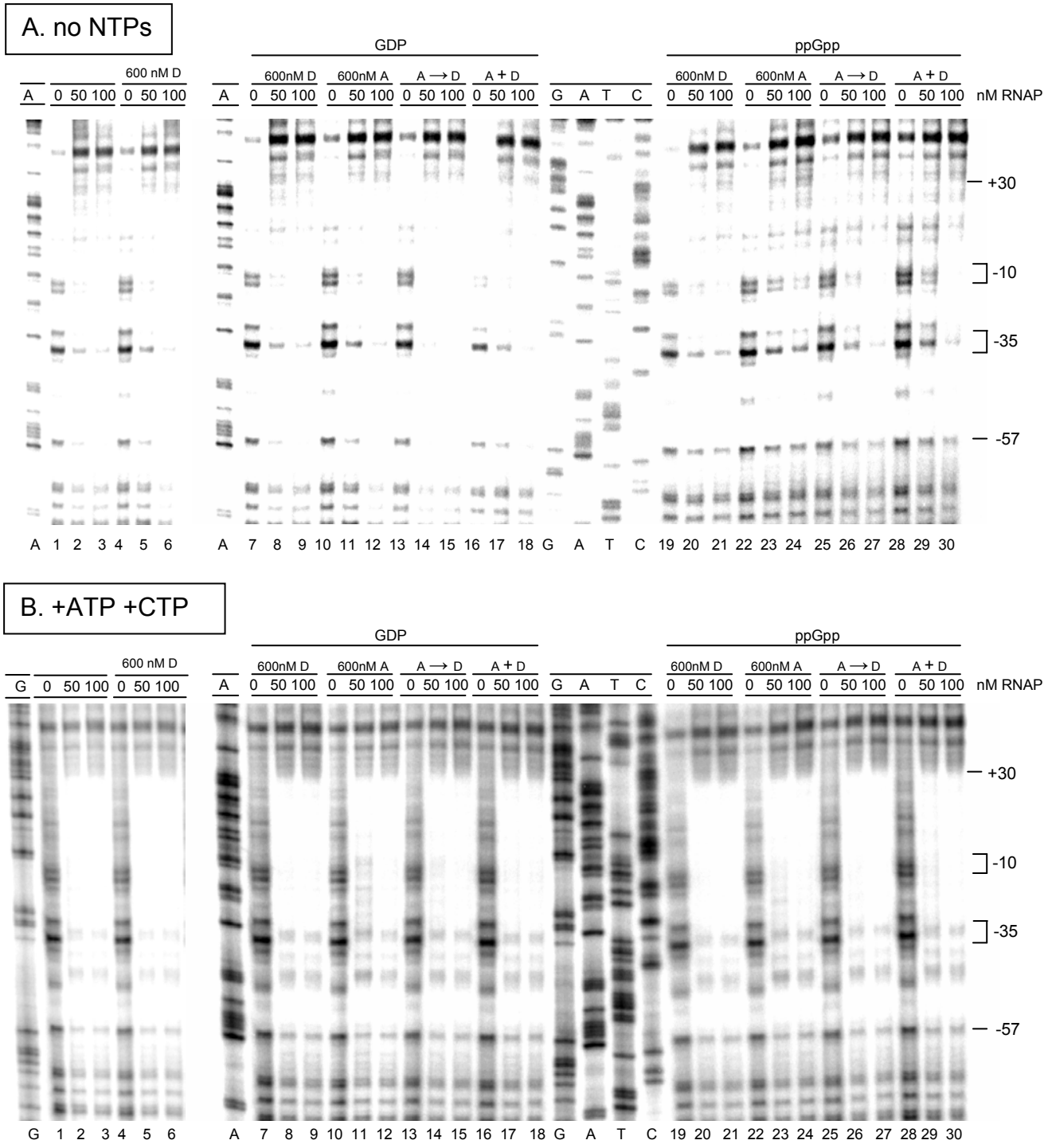

A. no NTPs
GDP
ppGpp
600 nM D
600nM D
600nM A
A → D
A + D
nM RNAP
+30
-10
-35
-57
B. +ATP +CTP

Fig.4.

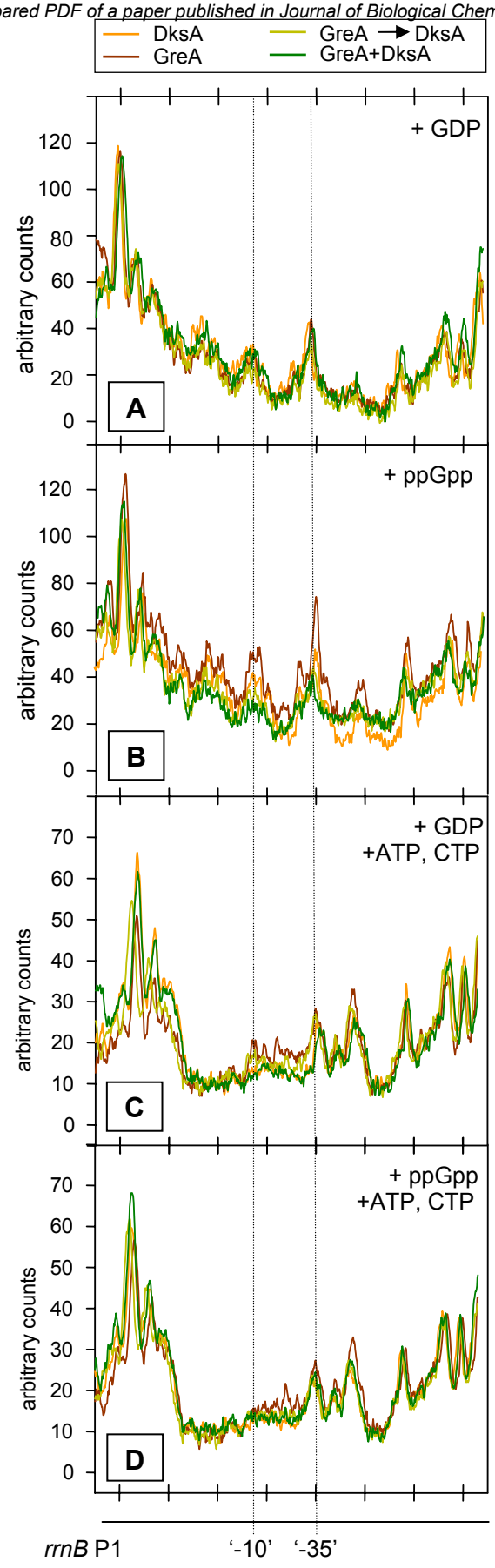

DksA
GreA
GreA → DksA
GreA+DksA
+ GDP
+ ppGpp
+ GDP
+ATP, CTP
+ ppGpp
+ATP, CTP
arbitrary counts
A
B
C
D
rrnB P1
'-10'
'-35'

Fig. 5.

This is an author-prepared PDF of a paper published in Journal of Biological Chemistry (2006) 281:15238-48

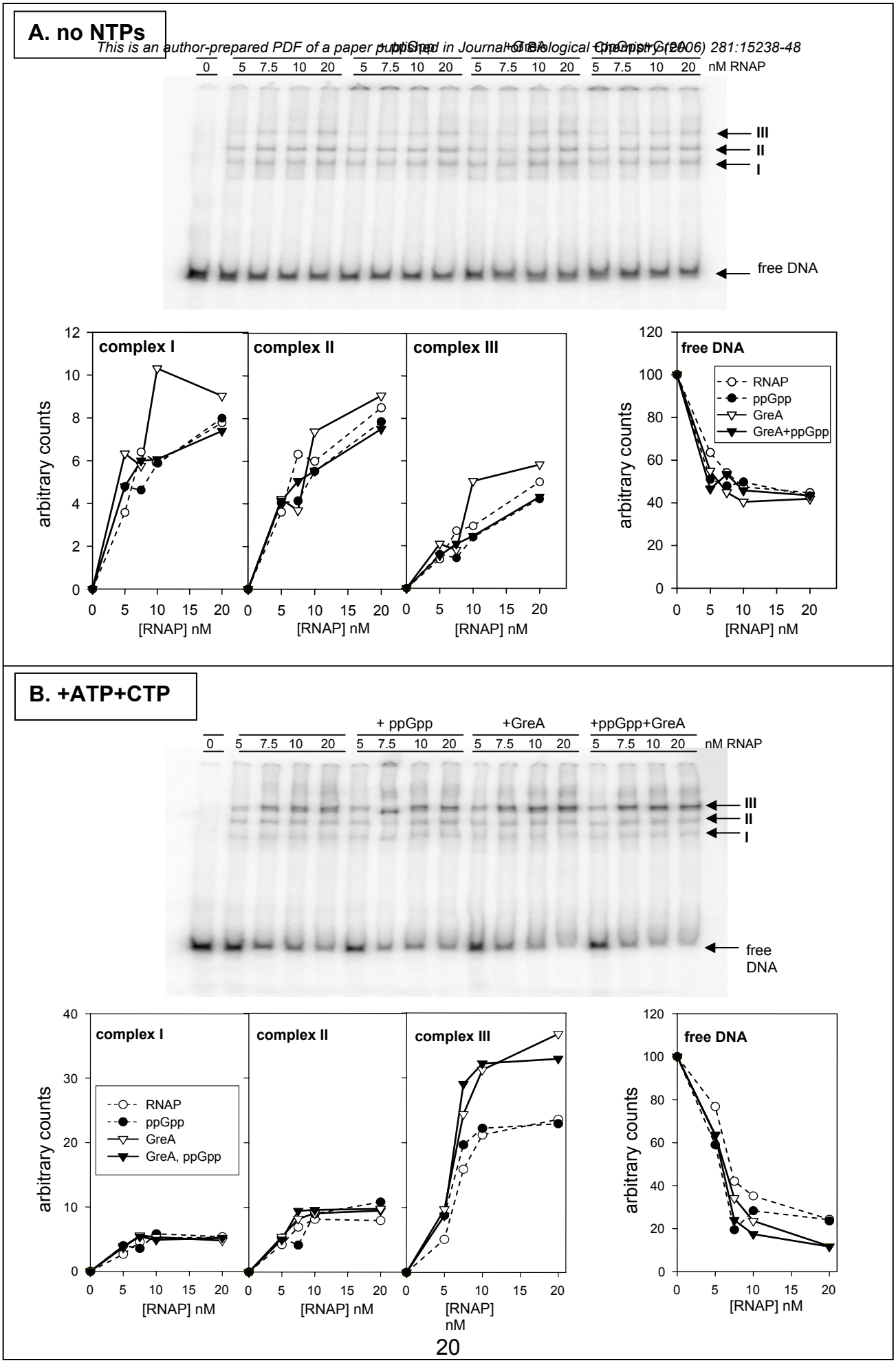
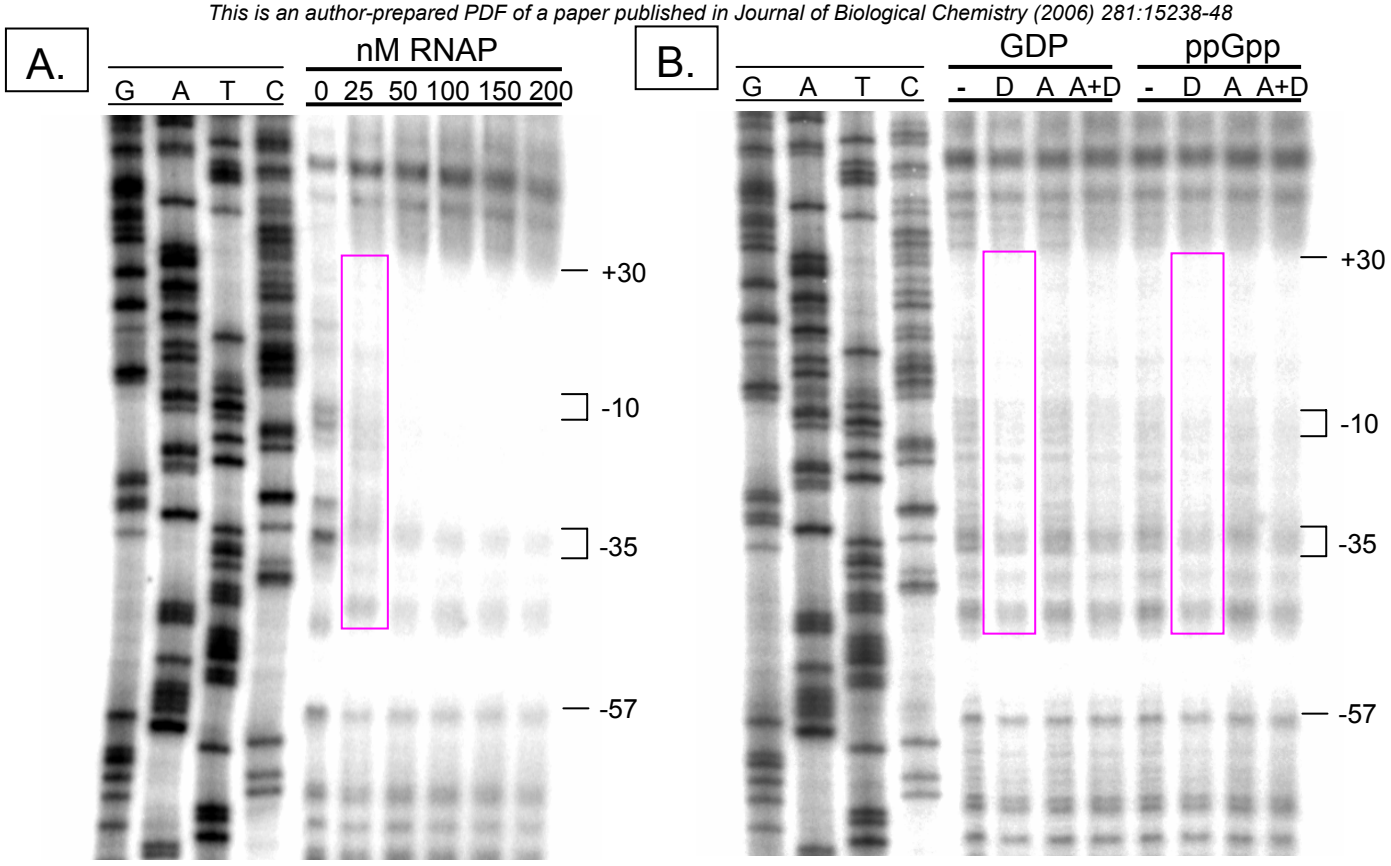


Fig.6

This is an author-prepared PDF of a paper published in Journal of Biological Chemistry (2006) 281:15238-48



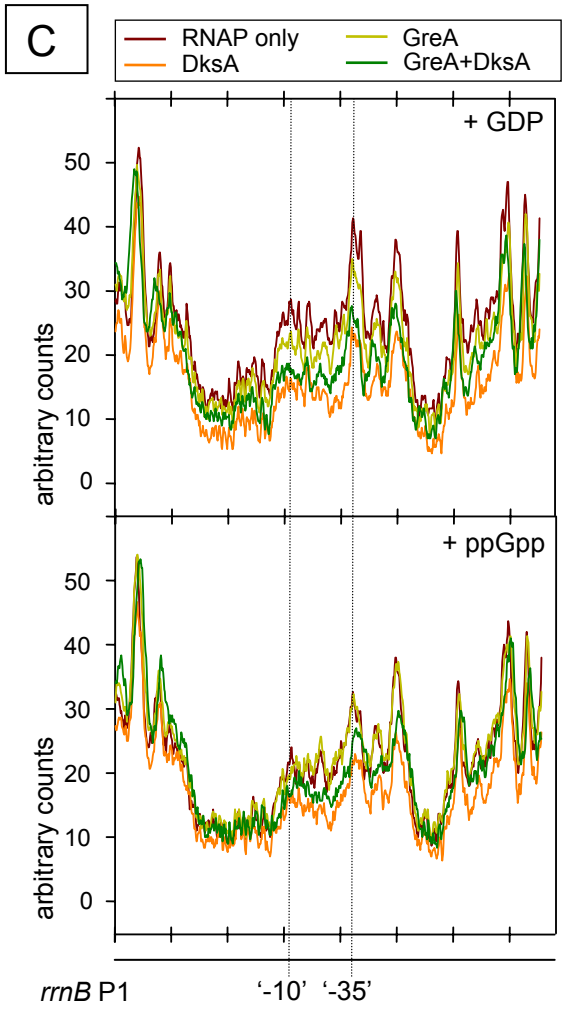


Fig.7.

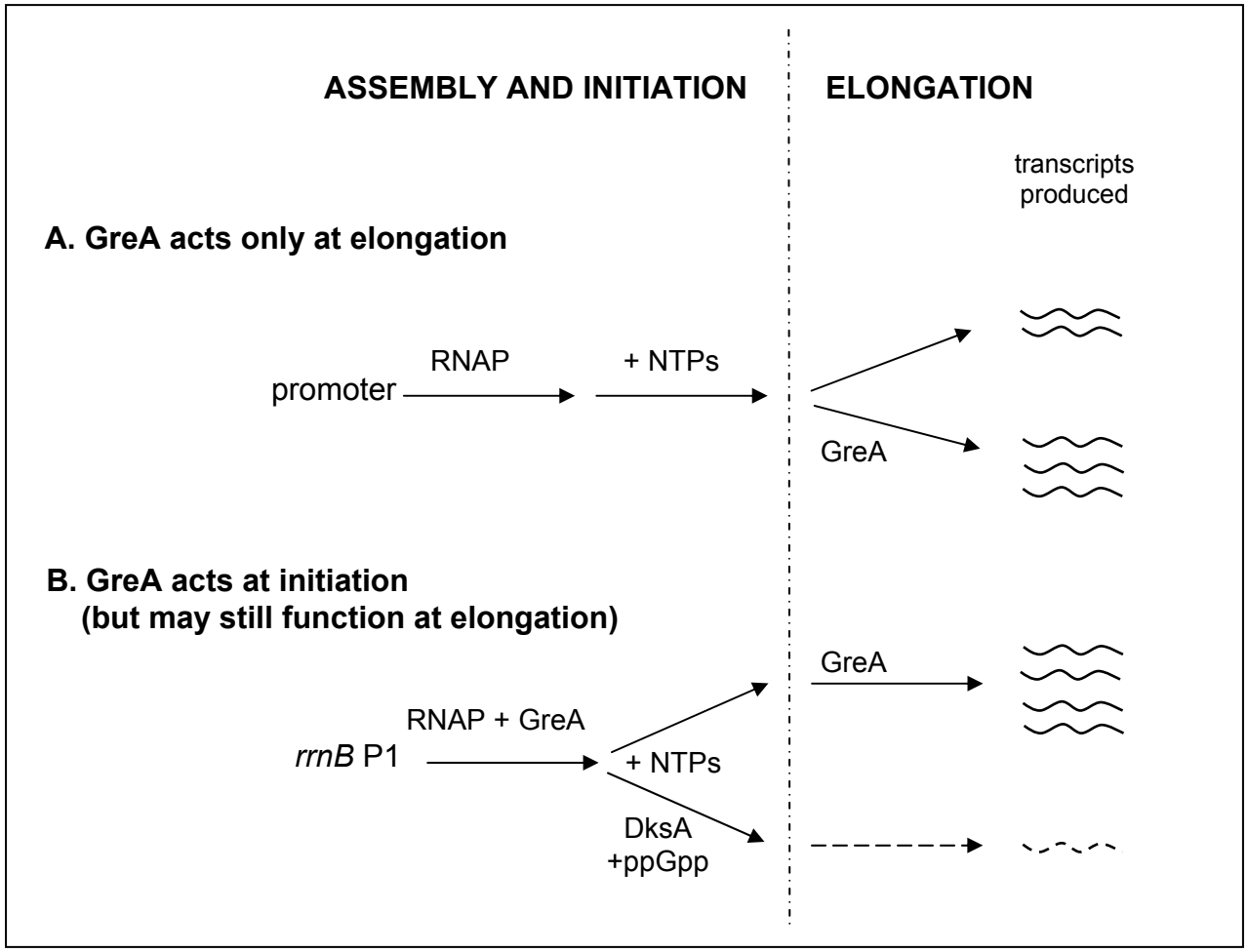

ASSEMBLY AND INITIATION
ELONGATION
transcripts produced
A. GreA acts only at elongation
RNAP
promoter
+ NTPs
GreA
B. GreA acts at initiation
(but may still function at elongation)
GreA
RNAP + GreA
rrnB P1
+ NTPs
DksA
+ppGpp

Fig. 8