

Transcription from bacteriophage λ pR promoter is regulated independently and antagonistically by DksA and ppGpp

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ABSTRACT

The stringent response effector, guanosine tetraphosphate (ppGpp), adjust gene expression and physiology in bacteria, by affecting the activity of various promoters. RNA polymerase-interacting protein, DksA, was proposed to be the co-factor of ppGpp effects; however, there are reports suggesting independent roles of these regulators. Bacteriophage λ major lytic promoter, pR, is down-regulated by the stringent response and ppGpp. Here, we present evidence that DksA significantly stimulates pR-initiated transcription *in vitro* in the reconstituted system. DksA is also indispensable for pR activity *in vivo*. DksA-mediated activation of pR-initiated transcription is predominant over ppGpp effects in the presence of both regulators *in vitro*. The possible role of the opposite regulation by ppGpp and DksA in λ phage development is discussed. The major mechanism of DksA-mediated activation of transcription from pR involves facilitating of RNA polymerase binding to the promoter region, which results in more productive transcription initiation. Thus, our results provide evidence for the first promoter inhibited by ppGpp that can be stimulated by the DksA protein both *in vivo* and *in vitro*. Therefore, DksA role could be not only independent but antagonistic to ppGpp in transcription regulation.

INTRODUCTION

Gene expression control in bacteria is often exerted at the level of transcription by employing global regulatory networks. Such systems are designated for precise and suitable adaptations of unicellular organisms to rapid environmental changes and the availability of nutrient sources. The stringent response effector, a specific nucleotide, guanosine tetraphosphate (ppGpp) is rapidly

produced in response to a variety of physico-chemical and nutritional stresses (1–3). ppGpp can directly and indirectly regulate expression of variety of genes, adjusting the survival of bacterial cells. It interacts with RNA polymerase (RNAP) near the active center (4); however, the exact binding site and determinants in the *Escherichia coli* enzyme remain controversial (5). Upon binding, ppGpp does not introduce any long-lasting conformational alterations to the enzyme (6). A major mechanism of ppGpp direct action at promoters was proposed to be based on the decreased stability of promoter–RNAP open complexes upon transcription initiation, for its both inhibitory (7) and stimulatory roles (8). In addition to the direct effect, ppGpp can influence the transcription capacity of RNAP by affecting the availability of core polymerase to σ factors in the cell (9,10) and controlling the synthesis of other transcription regulators (1,2,3,11). ppGpp is one of the most global and far-reaching regulators in bacteria: as shown by global transcription profiling in *E. coli*, stringent response involves alterations in expression of several hundred genes (12). However, in spite of over 40 years of studies, complete mechanism of the gene expression regulation by ppGpp remains not entirely solved (3). Significant and undisputable *in vivo* ppGpp effects, for example, were usually difficult to reproduce in purified *in vitro* system (3,11). The discovery of the DksA protein role resolved this discrepancy and was thus a mile-stone in understanding of the mechanism of the stringent response (13,14). DksA was shown to be a critical component for ppGpp-mediated inhibition of the ribosomal promoters *in vivo* (13,14). This 151 amino-acid protein interacts with RNAP binding in the enzyme's secondary channel (13,14) The effect of the concerted action of DksA and ppGpp is promoter-specific, depending on the intrinsic properties of a given promoter (3,15). For all promoters examined so far, DksA destabilizes competitor-resistance complexes between RNAP and promoter region (10,16). Recent studies propose that the mechanism of DksA-mediated regulation of transcription relies on allosteric alteration

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to the RNAP at the promoter, affecting the transition from closed to intermediate complexes (17,18). As DksA potentiates ppGpp regulation *in vivo* and *in vitro*, it was proposed to be a co-factor of the stringent response, enhancing its negative (e.g. ribosomal promoters) and positive (amino acids biosynthesis promoters) effects (13–15,19). However, several lines of evidence indicate that the roles of these transcription regulators can be opposing *in vivo*, when DksA could regulate gene expression without ppGpp (20), and independent *in vivo* and *in vitro* for the *fimB* promoter (21). Also, the transcriptomic analysis of ppGpp- and DksA-deficient strains indicates that certain genes (e.g. involved in chemotaxis) could be independently or differentially regulated by these factors (22).

In the prokaryotic world, expression of many genes is regulated simultaneously or sequentially by multiple factors, to obtain optimal adaptation to environmental challenges. Promoters of such genes can serve as model systems to study complex transcription regulation. Bacteriophage λ major lytic promoter, pR, can be controlled both positively and negatively by variety of phage and host factors, including λ -encoded Cro and CI proteins and bacterial regulators, DnaA and SeqA (23). Moreover, DNA topology alterations (mediated by IHF and potentially by other chromosome-organizing proteins) could affect pR promoter's activity (24). The stringent control alarmon, ppGpp, inhibits pR transcription both *in vivo* and *in vitro* (25–27). The correlation proposed by Barker *et al.* (7) and accepted in several other publications (13,16,28), between short life-time of competitor-resistance complexes and negative regulation by ppGpp, does not apply directly to pR, as its promoter-RNAP open complex is extremely stable (27). For pR, ppGpp was reported to affect the promoter escape step in transcription initiation, specifically, the first phospho-diester bond formation (27). At the time of publication of that work (2002), the DksA role in the stringent response was unknown, thus, the principle aim of this study was to elucidate a possible effect of DksA in pR-initiated transcription. Unexpectedly, we found that DksA does not act synergistically to the ppGpp effect; on the contrary, DksA directly stimulates pR-initiated transcription.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli MG1655 $\Delta lacZ$ strain (29), and its derivatives: ppGpp-null (*relA spoT*) $\Delta lacZ$ (from M. Cashel), *dksA* (RK201) (30) and the triple mutant *relA spoT dksA lacZ* were used in this work. Triple mutant *relA spoT dksA* was constructed by P1 transduction of the *dksA::Tn5* (kanamycin resistance) allele to the *relA spoT lacZ* strain. Cultures were routinely grown at 30°C in Luria broth (LB) (31) supplemented with appropriate antibiotics (kanamycin, 50 μ g/ml and ampicillin, 100 μ g/ml). Plasmid pTAC3734 (32), bearing a promoter-less *lacZ* reporter gene, was used for construction of pR-*lacZ* fusions as described by Łyżeń

et al. (33). For construction of supercoiled templates for *in vitro* transcription, plasmid pTE103 (34) was used as a vector, as described previously (33).

Nucleotides and proteins

Nucleotides were purchased from Roche Applied Science, (α - 32 P)UTP and (γ - 32 P)ATP from Hartmann Analytic GmbH, while ppGpp was synthesized and purified as described previously (34). The σ^{70} -RNAP holoenzyme was purified according to a general protocol described in (35) with modification as in (36). N-terminal His-tagged *E. coli* DksA was purified according to (21).

Measurement of β -galactosidase activity

Activity of β -galactosidase in *E. coli* cells was measured according to a previously described method (37). Multicopy gene fusions (located on plasmids) were used, thus the results were normalized per amount of plasmid DNA in cells; plasmid copy number in *E. coli* was estimated by isolation of plasmid DNA from a known number of cells, linearization with restriction endonuclease, separation during agarose gel electrophoresis, staining with ethidium bromide and subsequent densitometric analysis of the bands relative to a known amount of plasmid DNA separated on the same gel, as described in ref. (38).

In vitro transcription assay

Supercoiled template was obtained by isolation of pTE103-derived plasmid from wild-type MG1655 strain, and purification of supercoiled plasmid DNA by ultracentrifugation in CsCl-ethidium bromide density gradient (31). All *in vitro* transcription reactions were performed in general as described previously (24), with some modifications. The reactions were performed in a total volume of 25 μ l in the transcription buffer containing 50 mM Tris-HCl pH 8, 10 mM MgCl₂, 10 mM β -mercaptoethanol, 10 μ g/ml bovine serum albumin) with the final concentration of nucleotides: CTP and GTP of 150 μ M, ATP 1 mM, UTP up to 15 μ M and (α - 32 P)UTP up to 10 μ Ci with heparin (100 μ g/ml) for single-round transcription. Reactions were terminated by addition of stop buffer (150 mM EDTA, 1.05 M NaCl, 7 M urea, 10% glycerol, 0.0375% xylene cyanol, 0.0375% bromophenol blue). The samples were separated by electrophoresis in 4.5% polyacrylamide gel containing 7 M urea in the TBE buffer (22) at 30 mA. The gel was dried, and RNA bands were visualized and quantified using the PhosphorImager system (BioRad). For experiments as described in Figure 1A, RNAP (15 nM) was pre-incubated with the indicated amounts of the ppGpp and/or DksA protein for 7 min at the room temperature prior mixing with KCl (140 mM), followed by 3 min incubation in 37°C. After the addition of DNA (5 nM), the samples were incubated at 37°C for 10 min. The reaction were started by the addition of nucleotides (see above) with heparin (100 μ g/ml), the samples were incubated at 37°C for 10 min. The reaction was terminated by addition of 5 μ l of the stop buffer and analyzed as described earlier. For experiments as in Figure

1B, single-round transcription reactions were performed in a total volume of 17 μ l in transcription buffer (see above) containing 150 mM KCl. Template DNA (5 nM) with RNAP (15 nM), KCl (150 mM) and the indicated amounts of the ppGpp and/or DksA protein were incubated for 10 min at 37°C. After the addition of nucleotides (see above) with heparin (100 μ g/ml), the samples were incubated at 37°C for 10 min. The reaction was terminated by addition of 3 μ l of the stop buffer and analyzed as described earlier.

To analyze productive complex formation (Figure 4), the transcription reactions were carried out in transcription buffer (see above) supplemented by DNA template (5 nM) with KCl (150 mM) and the ppGpp (200 μ M) and/or DksA (400 nM) at 20°C for 10 min. The reactions were started by addition of RNAP (15 nM) and nucleotides mix (see above) with heparin (100 μ g/ml). At time-points, 17 μ l aliquots were removed and added to 3 μ l of the stop buffer and analyzed as described above. Figure 4 represents the transcription obtained at 3 min reaction time.

Open complex stability assay

RNAP (15 nM), DNA template (5 nM), KCl (150 mM) and ppGpp (200 μ M) and/or DksA (400 nM) were incubated for 10 min at 37°C in transcription buffer (see above). After heparin (100 μ g/ml) addition, 15 μ l aliquots were removed to a tube containing 2 μ l of nucleotides (see above), at indicated times (0, 30, 60, 90, 120, 150, 180, 240 min). Reactions were carried out by 10 min at 37°C. The reaction was stopped by addition of 3 μ l of the stop buffer and analyzed as above.

Electromobility shift assays

DNA linear fragments for RNAP *in vitro* binding studies were obtained by PCR using 5'-labeled (P^{32} primers: 5'-G CTCATACGTTAAATCTATCACCGCAAGGG and non-labeled 5'-GTAAGAGCGGGTTATTTATGCTG followed by subsequent purification of the 323 nt DNA fragment. DNA (15 ng) was incubated in B-buffer (25 mM HEPES, pH 7.5, 0.1 mM EDTA, 5 mM DTT and 10% glycerol) supplemented with 0.1 mg/ml poly(dI-dC), 80 mM KCl, 200 μ M ppGpp and/or 400 nM DksA and/or 1 mM ATP and UTP with increasing polymerase RNA concentration (0–10 nM) for 20 min in 37°C. The samples were separated on 3.5% Tris-glycine (pH 8.5) polyacrylamide gel at 120 V in 4°C. The DNA bands were visualized and quantified using PhosphorImager system (BioRad).

DNase I footprinting assay

End-labelled pR fragments (P^{32}) were obtained as for EMSA. Purification of the PCR products and subsequent footprinting analysis was performed as described in ref. (40), using 500 nM DksA, 400 μ M ppGpp and/or ATP and UTP at 1 mM and 40 nM RNAP.

RESULTS

DksA protein can activate transcription from pR *in vitro*

The stringent response and ppGpp overproduction inhibit pR activity; however, the mode of action of this effector is unusual comparing to other promoters (27). Generally, the *in vivo* effect of ppGpp on various promoters was usually difficult to reproduce *in vitro*. For both down-regulation of ribosomal RNA promoters and stimulation of amino acids biosynthesis promoters, DksA was shown to enhance the ppGpp effect in the reconstituted *in vitro* system. Thus, we aimed to test a hypothetical synergistic effect of DksA on ppGpp-mediated inhibition of the pR promoter. We employed the specific experimental set-up designed to study ppGpp effect *in vitro* (27), where ppGpp inhibited pR activity by about 30–40 % (Figure 1A). In all our *in vitro* transcription reactions, supercoiled template was used. This choice was justified by dependence of pR activity on the DNA topology (ref. 24 and our unpublished data). To our surprise, contrary to the anticipated results, DksA present in the *in vitro* transcription reaction stimulated pR activity over 3-fold at 800 nM DksA (Figure 1A). Moreover, the transcription was also enhanced when both regulators were present, at the constant level of ppGpp (200 μ M). It is worth mentioning that in these experiments single-round transcription assay was performed, thus, the DksA activation did not involve the re-initiation events. The specific assay, where ppGpp inhibition was initially observed, requires the RNAP to be incubated with ppGpp in low-salt buffer and room temperature, before other components, including salt, are added and temperature increases to the regular reaction level (37°C) (27). For other experiments, the usual set-up requires the salt to be present already in the reaction from the initial complex formation step. Therefore, we tested ppGpp and DksA effect in pR-initiated transcription in this experimental system. All components, including 150 mM KCl and buffer conditions, were the same as in the previous assay. In this case, we observed similar DksA-mediated stimulation of pR-initiated transcription (up to 3.5-fold); however, ppGpp inhibitory effect was negligible (Figure 1B). In the presence of both regulators, activation of transcription was observed only when DksA concentration increased at constant ppGpp level (200 μ M) (Figure 1). In the presence of increasing ppGpp concentrations, at constant DksA (400 nM), ppGpp has inhibitory effect on the transcription; however, one should note that the start-point is at 2.5-fold over the transcription with no addition and, even at the highest ppGpp concentrations, inhibited transcription was nevertheless 1.5-fold over the point where only ppGpp was present at the same concentration. The ability of DksA protein to inhibit transcription, as reported previously (13,14), was tested in the *in vitro* test employing the *rrnB* P1 promoter. In the experimental set-up as described in ref. (10), we observed a very similar inhibition of P1-initiated transcription (data not shown), indicating that this preparation of DksA protein retained its specific function.

Our results indicate that DksA can directly activate the pR promoter, alone and in the presence of ppGpp, in the

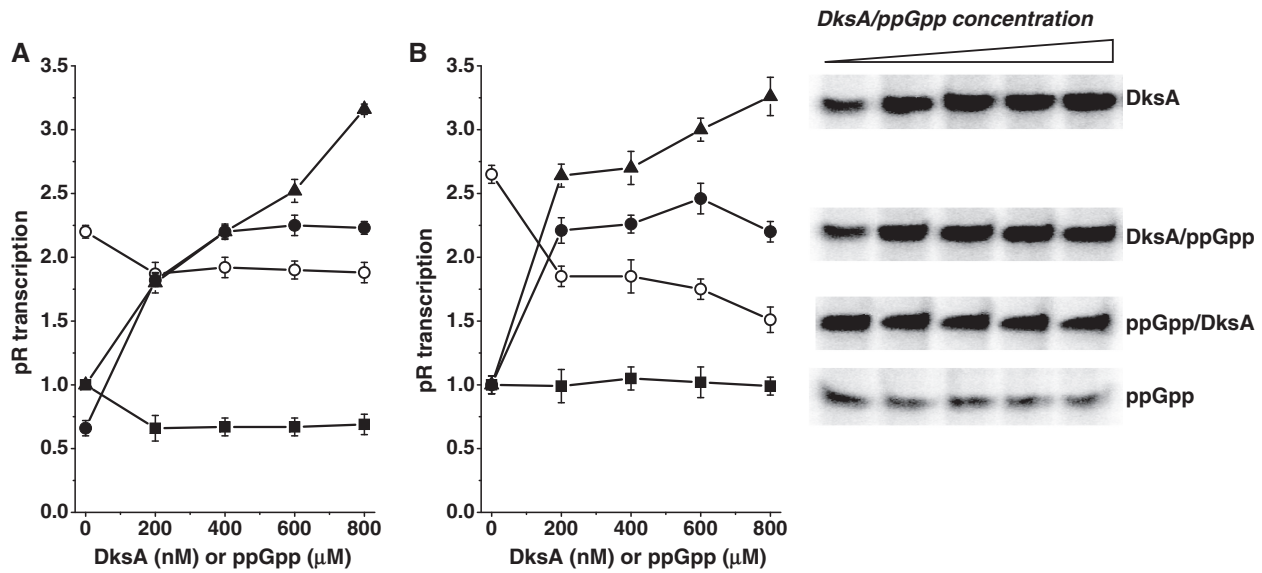


Figure 1. *In vitro* transcription from pR promoter in the presence and absence of ppGpp and DksA. (A) Transcription in the presence of increasing concentrations of ppGpp (squares), DksA (triangles), DksA with addition of 200 μ M ppGpp (closed circles) or ppGpp with addition of 400 nM DksA (open circles). RNAP and ppGpp/DksA were incubated 7 min at room temperature, then KCl was added (final concentration 140 mM) and temperature increased to 37°C prior to DNA addition. Transcription was initiated by adding of NTPs (see details in 'Materials and Methods' section). (B) Transcription in the presence of increasing concentrations of ppGpp (squares), DksA (triangles), DksA with addition of 200 μ M ppGpp (closed circles) or ppGpp with addition of 400 nM DksA (open circles). RNAP, DNA, ppGpp/DksA were incubated in the buffer with KCl (150 mM) in 37°C then reaction was initiated by adding of NTPs. Transcription in the absence of ppGpp and DksA was set as 1. Data are from three independent experiments with error bars indicated. Inset: Autoradiogram of transcription illustrating the example experiment performed as described in (B), corresponding to the plots.

experimental system allowing ppGpp-mediated inhibition of transcription. Thus, DksA can regulate pR transcription independently of ppGpp.

DksA is necessary for pR-initiated transcription *in vivo*

Elevated ppGpp level, due to the stringent response (e.g. by amino acid starvation) or RelA overproduction, resulted in the inhibition of pR-initiated transcription (25). Based on the finding that DksA can activate pR *in vitro*, we assessed the *in vivo* pR activity in the *dksA* mutant. A lack of DksA resulted in a significant decrease in pR activity, which was below 10% of that observed in the wild-type strain (Figure 2). The measurements were performed in the rich medium, in the exponential phase of bacterial growth. The pR activity in cells lacking both ppGpp and DksA was only slightly higher than in the *dksA* mutant, indicating that DksA role in pR-initiated transcription is prevailing at these growth conditions. The decrease in pR activity in cells lacking ppGpp (*relA spoT*), although surprising at first (given known ppGpp-mediated inhibition of pR), was already reported (26), where it was observed that low ppGpp levels are necessary for basal pR activity (Discussion section). These results suggest that DksA activity is indispensable for full pR activity *in vivo*. Taken together with the *in vitro* data, it can be concluded that DksA acts as an activator of pR.

DksA stimulates pR transcription by enhancing the binding of RNAP to DNA

To address the question about the molecular mechanism of DksA-mediated activation of pR-initiated transcription,

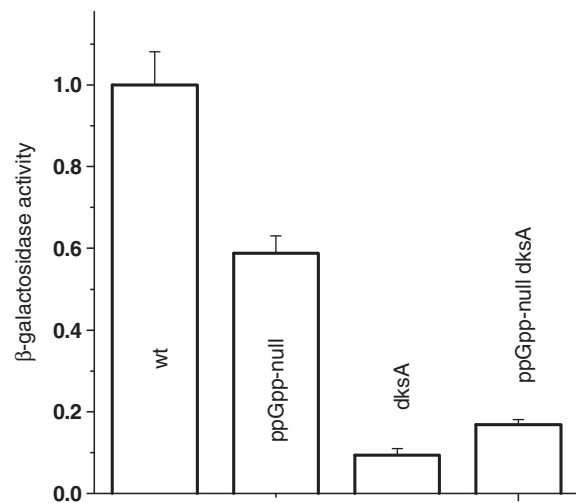


Figure 2. *In vivo* activity of pR promoter (β -galactosidase activity) in wild-type, *relA spoT* (ppGpp-null), *dksA* and *relA spoT dksA* strains. Activity in the wild-type (wt) strain was set as 1 (actual value was 12318 ± 1003 Miller units). The data are from six independent measurements with SD indicated.

we tested DksA effects at various steps in the transcription initiation. DksA was reported to decrease the stability of the competitor-resistant RNAP-promoter complexes for its both positive and negative regulatory effects (13,14,19). Thus, we assessed the effect of ppGpp and DksA on complex stability in the same as previously employed (Figure 1B) *in vitro* transcription system. Generally, pR forms extremely stable open complexes, with the half-life

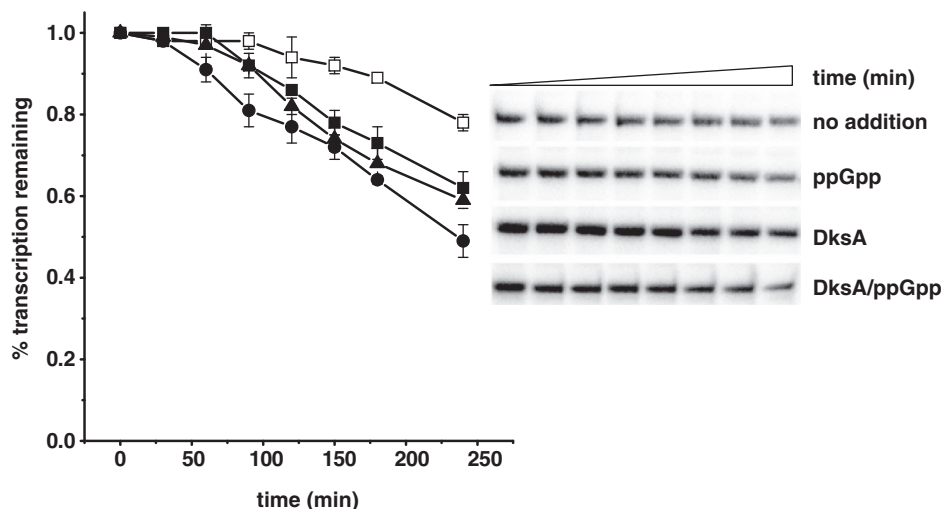


Figure 3. The effects of ppGpp and DksA on pR promoter half-life. (A) The time course of competitor-resistant open complexes was monitored by *in vitro* transcription challenged by heparin in the presence of none addition (DksA storage buffer, open squares), ppGpp at 200 μ M (closed squares), DksA at 400 nM (triangles) or DksA at 400 nM with addition of 200 μ M ppGpp (circles). The values found at 20 s after competitor addition were set as 1. Data are the average from three independent experiments with standard errors. (B) The autoradiogram from the experiment performed as shown in the (A).

time exceeding 6 h (extrapolation of the time-course plot and previous, unpublished observations). The presence of any of the regulators decreased the stability of the heparin-resistant complex down to about 4–4.5 h (Figure 3). However, the effect of DksA did not differ considerably from that of ppGpp on the complex stability. Moreover, the presence of both regulators did not result in further destabilization of complexes. Thus, we concluded that DksA does not act as a pR-transcription regulator by affecting the open complex half-life, although the protein itself destabilizes the complexes to the same extent as ppGpp. It was previously shown that ppGpp influences the promoter escape rate in transcription initiation (27). Therefore, we aimed to test a possible effect of DksA at this step of transcription. We found that DksA did not affect promoter escape, under the experimental conditions where ppGpp effect was observed (data not shown). At many bacterial promoters, RNAP produces certain amount of short transcripts, in addition to the full-length products, and this abortive transcription can influence the overall transcription efficiency. Thus, some regulators can affect this step in gene expression. However, the level of abortive transcripts measured in the different time points (including very short ones) in the presence of DksA was not altered, indicating that this cannot be a regulatory step involving DksA protein. The major effect of DksA in pR transcription was observed when in the *in vitro* transcription experiment RNAP was added at the same time as NTPs to the buffer–DNA reaction mixture containing also ppGpp/DksA (immediate start of transcription). In such experiments, the effects of regulators were evaluated at the steps of RNAP binding to DNA and productive initiation complex formation (the amount of the formed full-length transcript was assessed, Figure 4). The presence of DksA resulted in a significant increase in the amount of

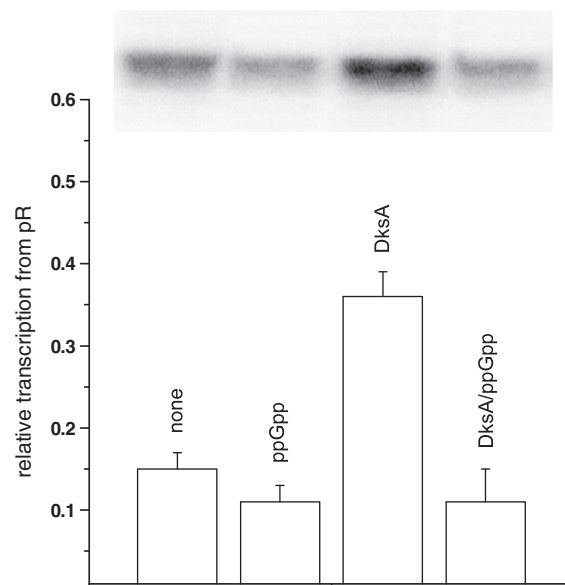


Figure 4. The effect of ppGpp and DksA on the *in vitro* transcription initiation. Relative transcription levels at 3 min after the reaction start (in the time-course experiment) Autoradiogram of transcription products is inserted above the columns representing transcription from pR. DNA was incubated with ppGpp/DksA in buffer containing 150 mM KCl with no addition, ppGpp at 200 μ M, DksA at 400 nM or DksA at 400 nM with addition of 200 μ M ppGpp. The reaction was initiated by simultaneous addition of RNAP and NTP with heparin, and samples were withdrawn at indicated times. Values were normalized to the level of transcription for 30 min with no addition (set as 1). The results are from three independent experiments with standard errors.

the transcript (2-fold effect) comparing to the transcription with no addition (Figure 4). Moreover, the time necessary for formation of full-length transcripts was shorter in the presence of DksA (the stimulatory effect

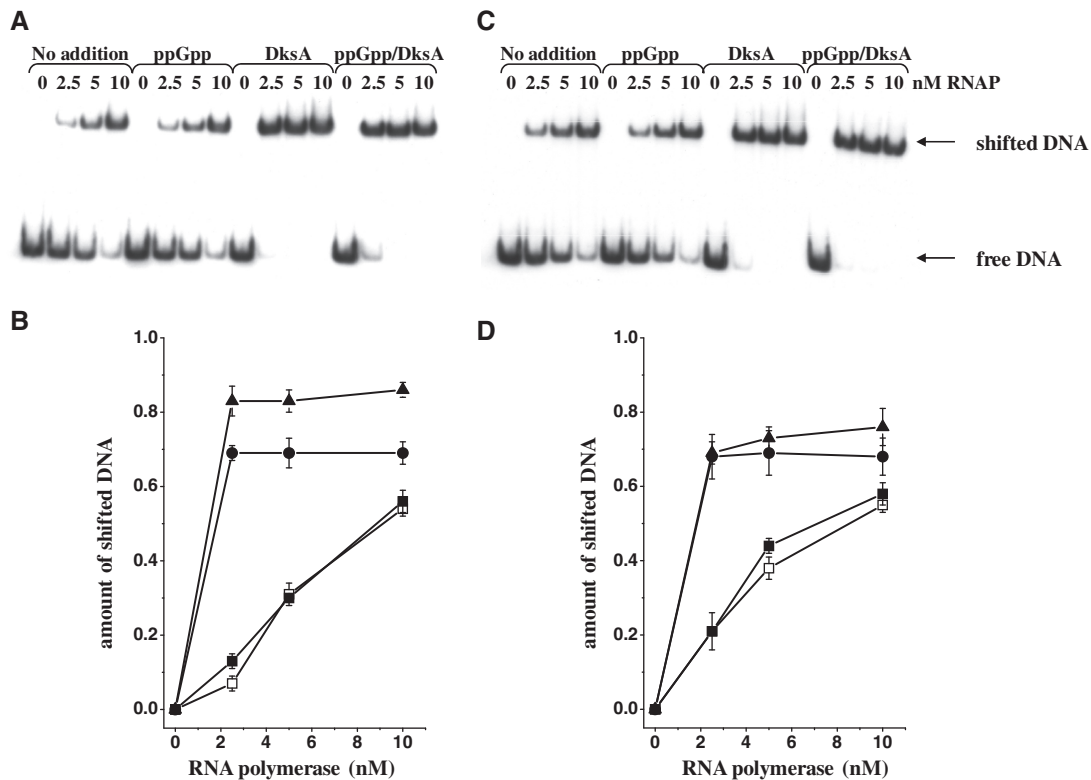


Figure 5. Binding of RNAP to pR DNA fragment. (A and C) Gel-shift assay using 15 ng of DNA and indicated amounts of the RNAP. (B and D) Quantification of the percentage of shifted (bound) DNA fragment. Symbols: none addition (DksA storage buffer, open squares), ppGpp at 200 μ M (closed squares), DksA at 400 nM (triangles) or DksA at 400 nM with addition of 200 μ M ppGpp (circles). In (C and D), ATP and UTP were present at 1 mM. The results are mean values from three measurements with error bars.

was observed already after 3 min). The slight inhibitory effect of ppGpp visible also in the presence of both regulators in longer reaction time (over 5 min) disappeared and DksA showed overall stimulatory effect on pR also in the presence of ppGpp (data not shown). These results indicate that pR activation by DksA occurs at the first step of the transcription initiation, i.e. RNAP interaction with the promoter region, which results in forming of the productive transcription complexes. The direct DksA-mediated stimulation of the RNAP binding to DNA was shown previously (21,41). These findings and our observation described above suggested that DksA-mediated pR transcription activation could be based on related mechanism. To test this hypothesis, we performed electrophoretic mobility shift assay (EMSA) of a pR promoter DNA fragment with increasing concentrations of RNAP and constant amount of DksA protein and/or ppGpp. As presented in Figure 5, DksA significantly stimulated RNAP ability to bind the promoter. DksA alone did not cause DNA shift (control experiment, data not shown). The presence of ppGpp did not result in any alterations in RNAP-DNA binding. However, when ppGpp was added together with DksA, the enhancement of RNAP-DNA interaction was slightly reduced (Figure 5B). This corresponds to the results presented in Figure 4, where addition of ppGpp decreased DksA stimulatory effect. The presence of two initiating nucleotides of the transcript, ATP and UTP, resulted in abolishing of

ppGpp effect at this step—at 2.5 nM RNAP majority of the DNA was visible as a complex with the protein (Figure 5C and D) for both DksA and DksA/ppGpp reactions. As EMSA experiment may have limitations due to the fragment linearity, problems in electrophoresis of the complexes and dissociation, we undertook another approach to study DksA role in RNAP binding to the pR promoter. The DNase I footprint analysis of the DNA fragment containing pR region showed more effective protection by RNAP in the presence of DksA. The binding was initiated by adding of RNAP and the effect was mostly visible at the short time—2 min (Figure 6); later, the footprint of RNAP could be too strong to visualize differences. The slightly weaker footprint of RNAP in the presence of both DksA and ppGpp occurred only when initiating nucleotides were absent; in the reaction where A+U were added RNAP bound very efficiently (data not shown, the protection was as strong as in the lane containing RNAP with A+U). The most pronounced DksA effect was visible when re-association of RNAP with pR was prevented by heparin. This set-up corresponds with single-round *in vitro* transcription, where DksA-mediated stimulation of pR was observed. However DksA increased the DNA protection by RNAP, the protection pattern and extent was not appreciably altered by DksA alone or with A+U.

We concluded from these *in vitro* data that the mechanism of DksA-mediated activation of pR-initiated

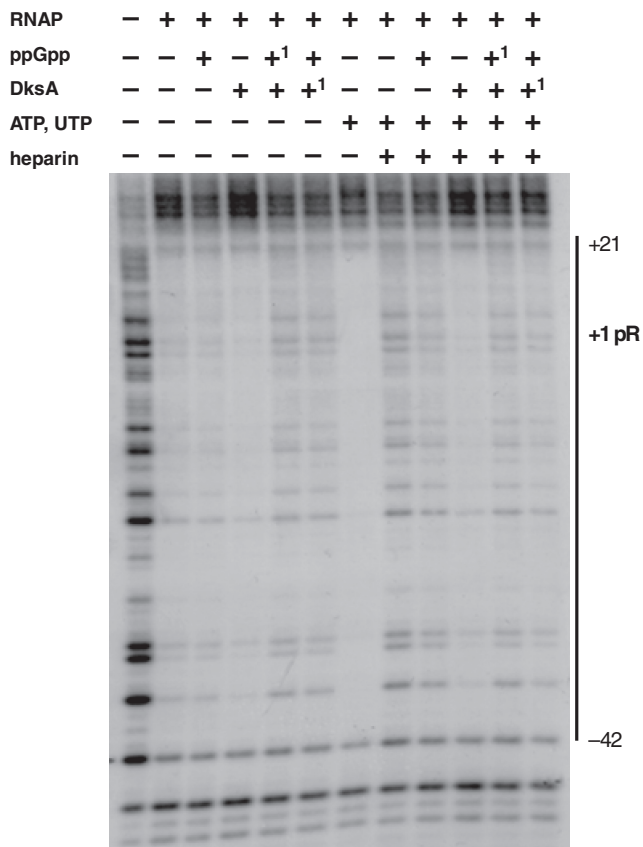


Figure 6. Footprinting analysis of RNAP protection at pR promoter in the presence of ppGpp and/or DksA. Reactions contained double-stranded template with the 5'-end (P^{32})-labeled, 40 nM RNAP, 400 μ M ppGpp, 500 nM DksA, ATP and UTP at 1 mM, heparin at 100 μ g/ml where indicated. In the lanes containing both DksA and ppGpp '1' indicates the regulator added as first.

transcription involves mainly facilitation of the association of RNAP with the pR promoter.

DISCUSSION

Discovery of DksA protein function in the stringent response, relatively recent comparing to the 40-years knowledge of ppGpp, elucidated its global role that initially was not expected when DksA was described as a suppressor of *dnaKJ* phenotype (30). From 2004, new aspects of DksA impact on gene expression as well as the approaches to explain the molecular mechanism of this regulation have been reported. Its importance for ppGpp-mediated transcription regulation has been documented in details (13,14,19), however unexpected roles of DksA are being recently revealed—as RNAP-binding protein DksA can affect transcription in the process independent on ppGpp (20,21).

Model promoter, bacteriophage λ -derived pR, is known to be down-regulated *in vivo* during stringent response and *in vitro* by its effector, ppGpp. In our work we aimed to elucidate the role of DksA in pR transcription. We present evidence that DksA does not facilitate ppGpp-mediated

down-regulation of pR activity. In contrary, our *in vitro* and *in vivo* results indicate that DksA acts as an activator of pR-initiated transcription. Under the same transcription reaction conditions where ppGpp evidently inhibits pR activity DksA can stimulate transcription from pR nearly 3-fold (Figure 1). In the set-up resembling *in vivo* situation (both regulators are present) DksA could stimulate pR at the low levels of ppGpp. However, increased ppGpp concentrations diminished DksA-mediated activation (Figure 1). Total transcription level, though, exceeded that observed without any regulator or with ppGpp alone. This supports stimulatory role of DksA in pR transcription. Our observation brought two main questions: what is the molecular mechanism of DksA-mediated activation of pR, and what is a possible role of this stimulation for physiological activity of pR in the context of phage–host interaction. To address first question, we investigated DksA impact on pR transcription initiation. For pR, as for all promoters tested so far, DksA can decrease competitor-resistant RNAP–DNA complex half-life (Figure 3); nevertheless, for the same promoter, it can significantly activate the transcription. The explanation of this inconsistency is discussed below. The detailed analysis of the transcription initiation excluded DksA effect at promoter escape step and the abortive transcription. The results presented in this work indicate that the main stimulation occurs at the step of RNAP binding to DNA. This DksA effect was reported recently for the *fimB* promoter (21). However, in the case of *fimB*, opposite DksA- and ppGpp-mediated regulation was observed only *in vivo*. The facilitation of RNAP binding to pR region by DksA was observed in EMSA and DNase I footprint assays. Interestingly, the presence of both regulators, ppGpp and DksA resulted in less effective binding only in the absence of initiating nucleotides. When the ternary complexes were created by first two phosphodiester bonds in the presence of ATP and UTP, ppGpp did not affect DksA-mediated enhancement of the binding. It could be speculated that this may correspond to the *in vivo* situation occurring upon starvation and stress, facilitating ppGpp effect when nucleotides are limited. Although DksA stimulated the RNAP binding to pR, it did not shift the endpoints of the DNase I protection, suggesting that the extent of the contact site of RNAP at pR is not influenced by DksA.

The discrepancy between destabilization of open complexes and activation of transcription by facilitation of the RNAP binding by DksA for the same promoter could be confusing at first. It appears that even if DksA decreases competitor-resistant complex life-time shifting the equilibrium into the dissociation direction for pR, the activation of the overall promoter activity occurs at independent level and the DksA effect depends on the intrinsic properties of a promoter as suggested previously (17,28). In the contrary to the activation of transcription from amino acids biosynthesis promoters by ppGpp/DksA, when stable complexes need destabilization in order to start effective transcription (12), ppGpp inhibits pR. Notably, presence of both regulators did not cause synergistical destabilization of RNAP–pR complex (Figure 3). The formation of transcription-competent

open complex of RNAP at pR involves at least two intermediates (42). The recent studies reported DksA-mediated allosteric alterations in RNAP during transcription initiation at *rrnB* P1, affecting the transition between intermediates (17). Whether DksA binding can result in the allosteric changes in RNAP at pR promoter, remains yet unsolved, however, one could speculate that if these alterations affect RNAP–DNA interface, this may enhance the effective binding of RNAP to promoter region. For the promoters forming stable complexes, this binding could be slow and require substantial energy, thus, enhancement at this step could facilitate overall promoter activity.

DksA can play a predominant role in pR regulation over ppGpp in the *in vitro* reconstituted system and in certain conditions of bacterial growth (discussed below). It is worth noting, that not only RNAP binding to DNA (which can be even inhibitory, if too strong), but also effective transcription (assessed by the formed transcript) is stimulated by DksA.

DksA is essential for pR transcription *in vivo*, with 10-fold decrease in pR activity in DksA-deficient *E. coli*. A lack of the anticipated effect of ppGpp-deficiency (an increase of the pR transcription in the absence of ppGpp would be expected), may be explained by the effects of cellular ppGpp concentrations. The reporter activity assays were performed in bacteria at exponential growth phase (at OD₅₇₅ of 0.2–0.3). Under these conditions, ppGpp level is the lowest relative to other growth rates. As was reported previously (26), low concentration of ppGpp can be stimulatory for pR, while a complete deficiency of ppGpp results in a decrease in pR-initiated transcription. Bacteria lacking both ppGpp and DksA supported pR activity to the level comparable to this observed in *dksA* strain, indicating that at least in the exponential growth phase, DksA is indispensable for effective pR-initiated transcription. The *in vivo* dependence of pR activity on DksA strongly indicates that the *in vitro* stimulation documented here is not an artifact of the specific experimental conditions in the reconstituted system.

Based on our observations, we therefore concluded that DksA plays important stimulatory role in pR transcription. Hence, DksA can be added to the list of λ pR regulators as a host-encoded activator among other important ones, such as DnaA or SeqA. This appends the new factor to the already complex model promoter. The transformation of λ plasmid is a function of efficient pR transcription due to transcriptional activation of *ori λ* . The transformation efficiency of the *dksA* strain was impaired (data not shown) suggesting that DksA has significant impact on the consequences of pR transcription in λ plasmid DNA replication.

To address the second question about the physiological role of the opposite regulation of pR transcription by ppGpp and DksA, one could hypothesize about its relation of the adaptation of λ phage development to host condition. λ , as a temperate phage, upon infection, can follow one of two alternative developmental pathways: lytic or lysogenic. The proper decision, supposed to ensure optimal phage propagation, has to

reflect the current physiological state of the host. Stringent response negatively regulates pR *in vivo*, indicating that ppGpp effect prevails in this specific situation. However, the absence of DksA results in the significant down-regulation of pR in the exponential growth. In addition to the situation when ppGpp is produced rapidly upon transition from nutrient availability (allowing fast growth) to starvation (stringent response), alarmon level increases significantly also in the situation of prolonged nutrient limitations (43). The latter situation is encountered by most bacteria (especially free-living prokaryotes) (44) and thus affects life cycle of bacteriophages infecting such host. Upon nutrient availability, phage lytic cycle would be preferable, thus, λ pR transcription responsible for synthesis of mRNA for replication proteins and crucial for transcriptional activation of *ori λ* , should be stimulated (26). This would explain multiple positive regulators of pR, and support the need for DksA while ppGpp level is low. In the situation when ppGpp concentration increases upon nutrient limitation, phage lytic development is inhibited (45); thus, pR transcription would be down-regulated, favoring lysogenic cycle. DksA-mediated up-regulation would be then taken over by ppGpp-mediated inhibition. Such a situation is mimicked in the *in vitro* experiment when ppGpp increases at the constant DksA level (Figure 1). However, the biological role of the sensitive balance of these regulators and their physiological functions leading to λ phage adjusting to environmental challenges of the host bacteria remain not entirely solved and are the subject of our current studies.

In summary, the results presented here shed a new light on the mechanisms of DksA and ppGpp effects during transcription regulation and give a new insight at pR transcription regulation. These data strongly indicate that although the stringent control requires DksA as a co-cofactor, this protein (by a different molecular mechanism) can play independent and antagonistic to ppGpp role in the regulation of gene expression.

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