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Properties of *Escherichia coli* RNA polymerase from a strain devoid of the stringent response alarmone ppGpp

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The stringent response alarmone guanosine tetraphosphate (ppGpp) affects transcription from many promoters. ppGpp binds directly to the transcription enzyme of *Escherichia coli*, RNA polymerase. Analysis of the crystal structure of RNA polymerase with ppGpp suggested that binding of this nucleotide may result in some conformational or post-translational alterations to the enzyme. These changes might affect *in vitro* performance of the enzyme. Here, a comparison of the *in vitro* properties of RNA polymerases isolated from wild type and ppGpp-deficient bacteria shows that both enzymes do not differ in i) transcription activity of various promoters (e.g. σ^{70}-*rrnB* P1, λpL, T7A1), ii) response to ppGpp, iii) promoter-RNA polymerase open complex stability. Thus, it may be concluded that ppGpp present in the bacterial cell prior to purification of the RNA polymerase does not result in the alterations to the enzyme that could be permanent and affect its *in vitro* transcription capacity.

Keywords: RNA polymerase, ppGpp, stringent response, transcription

INTRODUCTION

The regulation of transcription is a major control step in gene expression in all organisms. In the model bacterium *Escherichia coli*, RNA polymerase, a multi-subunit protein, exists in two forms: the catalytic core (α_2, β, β′, ω subunits) and the holoenzyme, with one of seven alternative σ-factors that confer promoter specificity upon transcription initiation (Burgess *et al.*, 1987). The transcription machinery usually is a target for modulation of its activity in accordance with the cellular requirements (for the most recent review, see Szalewska-Pałasz *et al.*, 2007a). The regulators controlling transcription can exert their function through a DNA binding or by influencing the transcription capacity of RNA polymerase without DNA contact. A number of regulators interacting with RNA polymerase belong to the latter group. The best known example are the stringent response alarmones, specific nucleotides: guanosine tetraphosphate (GDP 3′-diphosphate, ppGpp) and guanosine pentaphosphate (GTP 3′-diphosphate, pppGpp), collectively referred to as (p)ppGpp. This control system is one of the most far-reaching bacterial global regulatory signals employed to control cellular processes that would be energetically unfavorable during nutritional and physicochemical stress (reviewed by Shingler, 2003). A major role for (p)ppGpp is to balance the translational capacity under amino-acid starvation or limitation by mediating down-regulation of stable RNA (rRNA and tRNA) synthesis (reviewed by Cashel *et al.*, 1996). In *E. coli*, (p)ppGpp synthesis can be catalyzed by two paralogous enzymes: synthetase I (the *relA* gene product) and dual-function synthetase II (encoded by *spoT*) (Hernandez & Bremer, 1991; Xiao *et al.*, 1991; Gentry & Cashel, 1995). Double mutants of *relA* and *spoT* can not produce (p)ppGpp under any conditions, and are designated ppGpp-null strains (Cashel *et al.*, 1996). ppGpp and its recently discovered co-factor, DksA, regulate certain σ^{70}-dependent promoters (Paul *et al.*, 2004a; 2005; Perederina *et al.*,

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Abbreviations: DTT, dithiothreitol; ppGpp, guanosine tetraphosphate (GDP 3′-diphosphate); pppGpp, guanosine pentaphosphate (GTP 3′-diphosphate); rRNA, ribosomal RNA.

2004), exerting their function mostly during the transcription initiation, i.e. formation and stability of the RNA polymerase–promoter open complex and the formation of first bonds in the transcript. The effect may be either negative or positive depending on the specificity of a given promoter. ppGpp and DksA are also required for efficient *in vivo* transcription from promoters dependent on alternative σ-factors (Jishage *et al*., 2002; Laurie *et al*., 2003; Bernardo *et al*., 2006; Szalewska-Pałasz *et al*., 2007a).

The RNA polymerase is a subject of extensive structural and functional studies aimed at dissecting the specific mechanisms of regulation. The resolution of RNA polymerase crystal structure (Zhang *et al*., 1999) facilitated considerably the interpretation of results obtained in the course of studying the regulation of the enzyme activity. (p)ppGpp directly interacts with RNA polymerase to modulate its properties (Chatterji *et al*., 1998; Toulokhonov *et al*., 2001). Structural studies identified residues of the β- and β′-subunits accountable for ppGpp binding near the active site of the enzyme (Artsimovitch *et al*., 2004). However, a very recent publication (Vrentas *et al*., 2008) presents evidence that these particular residues may not be responsible for ppGpp binding to the *E. coli* enzyme. Binding of ppGpp to RNA polymerase does not cause major conformational changes; however, some alterations have been suggested, hypothetically mimicking those occurring during open complex formation (Artsimovitch *et al*., 2004). The association of ppGpp with RNA polymerase is relatively week, however, since the binding of ppGpp to RNA polymerase occurs frequently in the cell life, either during stress or normal physiological changes, e.g. stationary phase growth, it is plausible that RNA polymerase purified and employed in *in vitro* experiments could carry conformational alterations as an effect of the alarmone binding which could in turn influence its *in vitro* performance. In other words, the question arises whether the ppGpp-mediated changes in RNA polymerase conformation are long-lasting or rather transient. Another possibility could be post-translational modifications of RNA polymerase induced by its contact with ppGpp. Such alterations to the covalent structure of the protein could be relatively long-lasting and resulting in changes in its properties apparent after its biochemical purification. The evidences about post-translational modifications to RNA polymerase have been reported, either noncovalent (like binding of inorganic polyphosphate (Kusano & Ishihama, 1997) or RNA fragments (Sen *et al*., 2001)) or covalent (like proteolytic cleavage of the α subunit (Najmanova *et al*., 2003), ADP ribosylation of the α subunit (Rohrer *et al*., 1975; Goff, 1984) or phosphorylation of σ^{54} and β/β′ subunits (Jasiecki & Węgrzyn, 2006)). This work presents an attempt to answer these questions by comparing the biochemical properties of RNA polymerases originating from wild type and ppGpp-null strains of *E. coli.*

MATERIALS AND METHODS

Nucleotides, proteins and plasmids. Nucleotides were purchased from Roche Molecular Biochemicals. [α-^{32}P]UTP for *in vitro* transcription assays was from Amersham Bioscience or Hartmann Analytic. ppGpp was synthesized and purified as described by Cashel (1974). Components of the *dmpR*-pO *in vitro* transcription system: σ^{54}, IHF, DmpR-His were purified as described before (O'Neill *et al*., 2001; Sze *et al*., 2001). *E. coli* σ^{70} was purified essentially according to Fujita and Ishihama (1996) as described in Laurie *et al*. (2003). *E. coli* RNA polymerase was purified according to the general protocol described in Burgess and Jendrisak (1975) with modifications as from Hager *et al*. (1990). The wild type MG1655 and *relA spoT* strain (CF1693) were used for purification of RNA polymerase (Kvint *et al*., 2000). The DNA templates for *in vitro* transcription assays are presented in Table 1.

***In vitro* transcription.** Transcription assays were performed in a final reaction volume of 20 μl at 37°C in a buffer containing 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.275 mg/ml bovine serum albumin, essentially, as described before (Szalewska-Pałasz *et al*., 2007b). Briefly, for all transcription assays, core RNA polymerase (10 nM) was preincubated with appropriate σ factor for 5 min for holoenzyme association. The open complex formation was started by the addition of supercoiled DNA template, and, for σ^{54}-pO transcription, IHF (10 nM), DmpR-His (50 nM) and the aromatic effector 2-methyl-phenol (0.5 mM) and was then carried out for 20 min. Multiple-round transcription was initiated by addition of the NTPs mixture (for pO and pL transcription the final concentrations were as follows: ATP, CTP, GTP — 0.4 mM, UTP — 0.06 mM, [α-^{32}P]UTP — 5 μCi at 3000 Ci/mmol; for *rrnB* P1 transcription: ATP — 0.4 mM, CTP, GTP — 0.16 mM, UTP — 0.06 mM, [α-^{32}P]UTP — 5 μCi at 3000 Ci/mmol). The reac-

Table 1. Plasmids used in this work

Plasmid	Promoter	Reference
pCPG	σ^{70}-A1T7	Reynolds *et al*., 1992
pVI901	σ^{70}-λpL	Szalewska-Pałasz *et al*., 2007b
pRLG6214	σ^{70}-*rrnB* P1	Schneider *et al*., 2002
pVI695	σ^{54}-*dmp* Po	Laurie *et al*., 2003

tion was continued for 5 min and then heparin was added to the final concentration of 0.1 mg/ml to prevent reinitiation. After further incubation for 5 min the reactions were stopped by adding 5 μl of stop/load buffer (150 mM EDTA, 1.05 M NaCl, 7 M urea, 10% glycerol, 0.0375% xylene cyanol, 0.0375% bromophenol blue). For single-round transcription, heparin at 0.1 mg/ml was present in the NTPs mixture and the reaction proceeded for 10 min followed by addition of the stop/load buffer. Transcription products were then analyzed on 7 M urea, 4.5% or 6% (for *rrnB* P1) polyacrylamide gel and quantified by phosphorimaging.

Open complex stability assay. The half-life of open complexes formed on specific promoters was assessed in the *in vitro* transcription assay as described above. The complexes, after pre-forming, were challenged by a competitor and at indicated times aliquots of 20 μl were withdrawn and the single round transcription (described above) was performed to measure the functional complexes. For λ-pL and *dmp*-pO, heparin was used as a competitor (at the concentration of 0.1 mg/ml) and for the highly unstable σ^{70}-*rrnB* P1 promoter complexes, double-stranded competitor DNA was used (Gaal *et al*., 2001).

RESULTS AND DISCUSSION

Purification of the RNA polymerase from wild type and ppGpp-null strains

The native enzyme preparation was done according to the standard procedure (Burgess & Jendrisak, 1975; Hager *et al*., 1990). Proteins isolated from exponentially growing bacteria were purified in two steps: first, on a DNA-agarose column, employing the ability of RNA polymerase to bind DNA, and then on an anion-exchange Mono-Q column. The latter step allows the separation of the holoenzyme and the core. A comparison of the protein profiles at both steps of the purification indicates that both preparations do not differ in their abilities to bind DNA and the proportion of the holoenzyme to core in the final purification step is very similar (Fig. 1). The latter observation may indicate that the intrinsic ability to form the σ^{70}-holoenzyme is not affected by the presence of ppGpp. All this suggests that the basic properties of the enzymes isolated from wild type and ppGpp-null strain are not significantly different. The purifications were done three times independently, and all subsequent analyses were performed using different preparations giving reproducible results.

Comparison of *in vitro* transcription activity of RNA polymerases from different strains

The activity of the polymerase during transcription from different promoters is dependent on many factors, e.g. promoter sequences, regulators, availability of transcription components. Various promoters can respond differently to the stringent response alarmone ppGpp. As an altered activity of the polymerase isolated from the strain devoid of ppGpp could be expected, an assortment of promoters was selected exhibiting different responses to the stringent control alarmone. The rRNA *rrnB* operon P1 promoter is under a widely documented negative influence by ppGpp (Paul *et al*., 2004b), while both bacteriophage promoters, λpL and T7A1, are not responsive to ppGpp. The alternative σ-factor promoter, pO of the *dmp* operon, transcribed by σ^{54}-holoenzyme, is strictly dependent on ppGpp *in vivo* (Sze & Shingler, 1999). Assuming that the RNA polymerase purified from the wild type strain is conformationally different from the one that has never encountered ppGpp, one would expect that these enzymes may have different transcription abilities on promoters influenced by ppGpp. For this, the transcription from *rrnB* P1 would be an indicator. The *in vitro* transcriptional activity of various promoters in the presence of either type of the enzyme was compared by σ^{70} titration (Fig. 2). The results indicate that the transcription activities of P1 and the neutral to ppGpp pL and A1 promoters are very similar for both polymerases (Fig. 2A, B). A minor

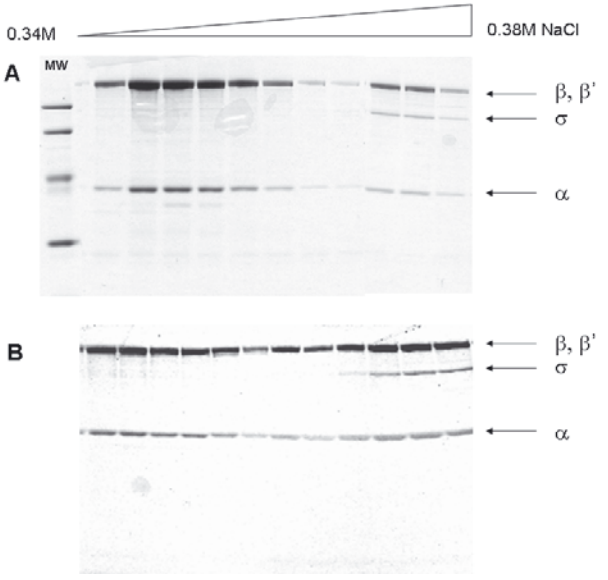

Figure 1. Purification of RNA polymerase from wild type (A) and *relA spoT* (B) strains.
Fractions were collected from 8 ml MonoQ column, in the 0.34–0.38 M NaCl gradient. Positions of core and holoenzyme subunits are indicated by arrows. MW — molecular weight standard (97, 66, 45, 30 kDa).

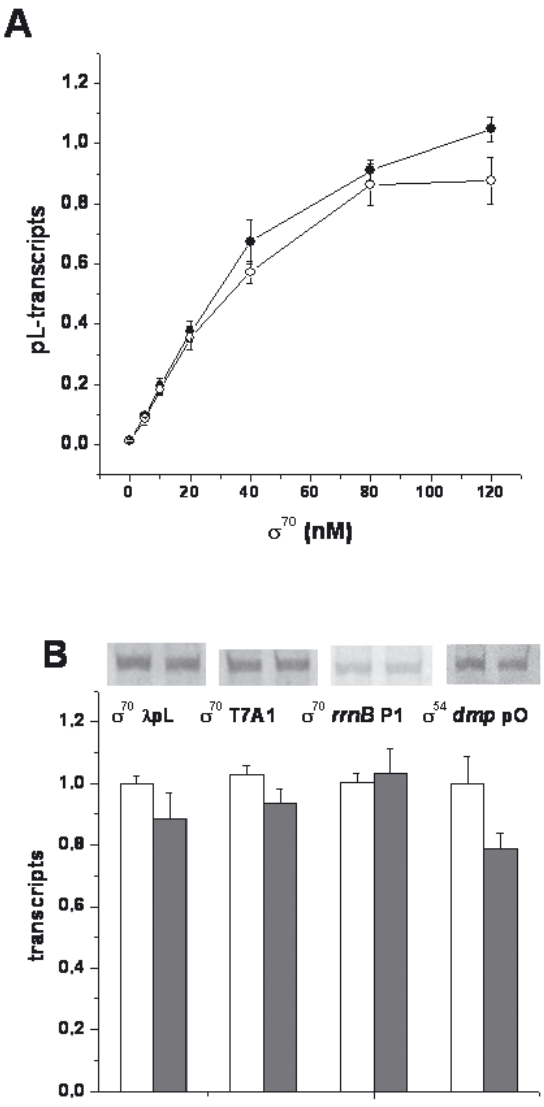

Figure 2. Relative σ^{70} and σ^{54} transcription by wild type and ppGpp-null RNA polymerases.
A. Multiple round titration of the core with increasing concentrations of σ^{70} on pL promoter, wild type polymerase — closed circles, ppGpp-null polymerase — open circles; B. Corresponding relative levels of transcription with 10 nM core (wild type — empty columns, ppGpp-null — shaded columns) and 80 nM of indicated σ (for *rrnB* P1, 100 nM). Transcription by wild type enzyme was set as 1 for each promoter. Inset: examples of transcripts from specific promoters employing wild type or ppGpp-null enzymes corresponding to the columns. Data are the average of three independent experiments with standard errors.

(less than 20%) difference was observed only for the σ^{54}-pO transcription (Fig. 2B). Taking into account that the *in vivo* pO activity in the absence of ppGpp is below 10% of that observed in the wild type strain (Sze & Shingler, 1999), the *in vitro* results indicate that any conformational or post-translational changes brought about by the presence of ppGpp may play only a marginal role for this transcription.

The proposed mechanism of the indirect and passive influence of ppGpp together with DksA on σ^{54} transcription *in vivo* (Bernardo *et al.*, 2006) explains also why no significant changes in the transcription level could be observed in the case of wild type and ppGpp-null RNA polymerase. For such analysis, a comparison of the activity of a promoter directly affected by ppGpp would be more meaningful. If the potential conformational change due to the presence of ppGpp could influence the transcription, then in the case of ppGpp-free RNA polymerase one would expect elevated transcription from a promoter inhibited by ppGpp, such as P1. The lack of any notable differences in the transcription from P1 as well as the control phage promoters indicate that the RNA polymerase purified from the ppGpp-null strain does not exhibit altered intrinsic features in the transcription from the promoter affected by ppGpp.

ppGpp responsiveness of RNA polymerases isolated from wild type and ppGpp-null strains

The RNA polymerase interacts directly with ppGpp (Artsimovitch *et al.*, 2004). The pool of the enzyme can be bound with this nucleotide, depending of its cellular level. This level varies from very low in the exponential growth phase to an elevated level upon entrance into stationary phase and a dramatic increase upon starvation or stress. Thus, purification of RNA polymerase may result in isolation of some pool of enzyme associated with ppGpp or with some conformational or covalent changes. All this would result in an altered response to ppGpp *in vitro* e.g. an enzyme that has not come previ-

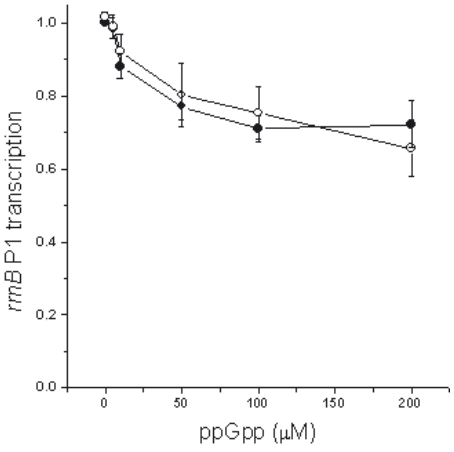

Figure 3. *In vitro* responsiveness to ppGpp.
Multiple round transcription from *rrnB* P1 promoter with appropriate core RNA polymerase (wild type — closed circles, ppGpp-null — open circles) with increasing concentrations of ppGpp. Transcription in the absence of ppGpp was set as 1 for each polymerase. Data are the average of three independent experiments with standard errors.

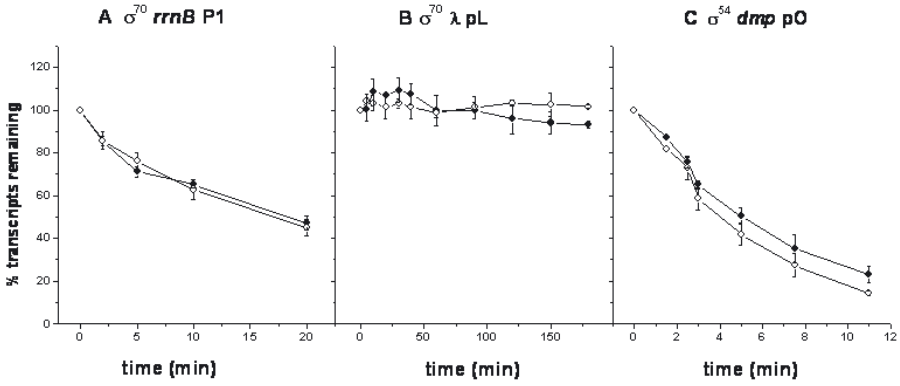

Figure 4. RNA polymerase–promoter open complex stability.
The time course was monitored by *in vitro* transcription in the presence of competitor (heparin for *dmp*-pO and λpL, double-stranded DNA for *rrnB* P1), wild type polymerase — closed circles, ppGpp-null polymerase — open circles. Note the difference in the time scale. Data are the average from three to five independent experiments with standard errors.

ously in contact with ppGpp could exhibit a stronger response. Therefore, an attempt to elucidate the ppGpp responsiveness of the enzyme purified from strain devoid of ppGpp was undertaken. The *in vitro* inhibition of the *rrnB* P1 promoter by ppGpp was tested. The results (Fig. 3) indicate that the ppGpp sensitivity of the two enzyme preparations does not differ significantly. Similarly, the pO activity was not affected by ppGpp in *in vitro* transcription in the presence of either enzyme (not shown). For pO, no effect on the *in vitro* transcription was observed for the wild type enzyme, as reported previously in Laurie *et al*. (2003); therefore it was important to test the possible effect of ppGpp on the polymerase that has never contacted ppGpp. The lack of a ppGpp effect indicates that, as suggested earlier, the major *in vivo* effect of ppGpp is indirect (Laurie *et al*., 2003; Bernardo *et al*., 2006). The presented observations show that the RNA polymerase purified from ppGpp-null strain does not differ in its response to ppGpp from the wild type enzyme.

Stability of the open complexes formed at σ^{70} and σ^{54} promoters by RNA polymerases

One of the most important stages in transcription initiation where ppGpp exerts its function is the formation and stability of the open complex of RNA polymerase and promoter DNA (Bartlett *et al*., 1998; Barker *et al*., 2001). The effect of destabilization of these complexes depends on the promoter: for the highly unstable rRNA promoters, further destabilization dramatically reduces the transcriptional output, while promoters known to be stimulated by (p)ppGpp are further activated through more efficient isomerization from closed to open complexes (Paul *et al*., 2004a; 2005). The destabilization, however, occurs even for promoters not affected by ppGpp, e.g. λpL (Szalewska-Pałasz *et al*., 2007b). Thus, if the RNA polymerase purified from the strain devoid of ppGpp could exhibit any different behavior from the wild type, it would occur most likely at this step of transcription. To test this hypothesis, the open complex stability was assayed on the templates of *rrnB* P1, λpL and σ^{54}-pO promoters with the competitor challenge. The stability was very similar for both polymerases for all the promoters tested (Fig. 4). Moreover, the half-life of the complexes corresponds to the one observed previously for this type of challenge (Szalewska-Pałasz *et al*., 2007b). The results demonstrate that the ability of RNA polymerase to form and maintain the open complexes is not affected by the presence of ppGpp in the bacteria that were the source of the enzyme. This supports the previous suggestions that ppGpp does not introduce permanent alterations that could withstand the purification procedures.

CONCLUDING REMARKS

The *in vitro* experimental conditions are usually designed to imitate the situation in the living cell as closely as possible. Therefore, the proper choice of components used in the *in vitro* studies is crucial for meaningful interpretation of the obtained results. Thus, the comparison of the properties of RNA polymerase purified from a wild type and a ppGpp-deficient strain was aimed to address the question whether the presence of the stringent alarmone could affect the basic features of the enzymes, and particularly, whether ppGpp-mediated alterations to the RNA polymerase structure could be long-lasting or only temporary. This work demonstrates that the steps that are under ppGpp influence, i.e. the transcriptional output from various promoters, and the stability of the initial open complexes are very similar for both polymerases. Thus, it could be conclud-

ed that ppGpp (at least at the level present in exponentially growing cells) does not result in alterations of RNA polymerase that could be sufficiently stable to affect its *in vitro* transcription capacity.

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