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The differential effects of guanosine tetraphosphate on open complex formation at the *Escherichia coli* ribosomal protein promoters *rplJ* and *rpsA* P1

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Abstract

The effects of guanosine tetraphosphate (ppGpp) on inhibition of single-round in vitro transcription and on the kinetics of open complex formation were investigated at the *Escherichia coli* ribosomal protein promoters *rplJ* and *rpsA* P1. The two promoters differ in their saturation characteristics and sensitivities to ppGpp. With a 10:1 molar ratio of RNA polymerase (RNAP) to DNA, saturation of transcription activity and weak inhibition ($\approx 30\%$) are observed at *rplJ*, in contrast to the weak activity and strong inhibition ($\approx 80\%$) at *rpsA* P1. In the absence of ppGpp, the two promoters show a threefold difference in the overall rate constants of association (k_a) (6.5×10^7 M^{-1} s^{-1} at *rplJ* and 2.0×10^7 M^{-1} s^{-1} at *rpsA* P1), while the dissociation rate constants (k_d) are similar ($\approx 4.8 \times 10^{-5}$ s^{-1}). The addition of ppGpp causes a twofold reduction in k_2 (isomerisation constant) at *rplJ* and a threefold decrease in K_B (equilibrium constant of RNAP binding) at *rpsA* P1. There is a significant twofold increase in k_d at *rplJ*, compared with smaller changes at *rpsA* P1 and at the non-stringent *lacUV5* promoter. These results indicate that ppGpp affects the formation and stability of the open complex at the *rplJ* promoter, in contrast to the inhibition of RNAP binding to the *rpsA* P1 promoter.

Keywords: ppGpp; Ribosomal protein promoters; Stringent response; Open complex

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Abbreviations: ppGpp, guanosine tetraphosphate; RNAP, RNA polymerase; dsDNA, double-stranded DNA; NC, nitrocellulose; DEAE, diethylaminoethyl; RP_c, closed complex; RP_o, open complex; [RNAP], RNA polymerase concentration; [RNAP]/[DNA], ratio of RNAP to DNA concentrations; nt, nucleotide

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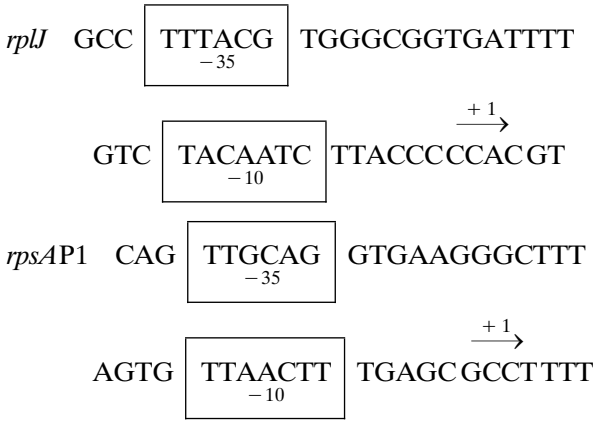
1. Introduction

Rapidly growing *Escherichia coli* cells that are subject to amino acid deprivation, undergo a panoply of metabolic and physiological changes collectively termed the Stringent Response [1]. Two critical regulatory events underlying these changes are the selective inhibition of ribosomal RNA/protein synthesis and the concomitant stimulation of biosynthesis of various metabolic precursors. These processes are mediated either directly or indirectly by the regulatory nucleotide guanosine tetraphosphate (ppGpp) [2,3], but the precise mechanism by which ppGpp exerts differential effects on the regulation of gene expression still remains unresolved [1].

The finding that mutations in RNA polymerase subunit genes alter the sensitivity of stable RNA synthesis to intracellular ppGpp levels, showed that RNA polymerase (RNAP) is a target for ppGpp, thereby suggesting that the negative control of rRNA and tRNA (stable RNA) synthesis by ppGpp occurs primarily at the transcriptional level [4]. The direct interaction between RNAP and ppGpp has been demonstrated with the report of a distinct ppGpp-binding site on the holoenzyme [5].

The effects of ppGpp on *rrn* transcription have been attributed to influences on various steps of the initiation reaction: inhibition of promoter binding [6–8], destabilization of open complexes [9], and the slow conversion of closed to open complexes [10]. Other studies report that the inhibitory effects of ppGpp are due to enhanced site-specific pausing at the *rrnB* promoter in vitro [11–13] and decreasing elongation rates in vivo [14].

In contrast to the extensive studies carried out with stable RNA promoters, very little is known about the functional properties or stringent control of ribosomal protein promoters. The transcription of many ribosomal protein genes are known to be stringently regulated [15–17]. Mixed-template, single-round in vitro transcription assays, have shown that ppGpp strongly inhibits transcription from two ribosomal protein promoters, *rplJ* and *rpsA* P1, presumably by affecting the stability of the open complex [9]. Both promoters contain a GC-rich discriminator motif and non-consensus −35 sequences, as shown below, but have not been tested for their in vivo response to stringency [18].



The *rplJ* promoter whose sequence is shown above, drives the expression of the *rplJL* and *rpoBC* genes that code for ribosomal proteins L10, L7/L12 and RNA polymerase subunits β and β', respectively [19]. It is one of the few known *E. coli* promoters that initiate with a pyrimidine nucleotide [20] and although it is relatively strong in vivo [21,22], it is very sensitive to salt and temperature in vitro [23]. The *rpsA* gene encoding the ribosomal protein S1, is transcribed from two major promoters P1 and P3 separated by approx. 130 bp [24]. The upstream P1 promoter whose sequence is shown above, shows weak activity and strong inhibition by ppGpp, but is relatively insensitive to salt and temperature [9,25]. Considering that the sensitivity of stringent promoters to salt and RNAP concentrations, influences the inhibition by ppGpp [18], the contrasting properties of the *rplJ* and *rpsA* P1 promoters necessitate a detailed study of their interaction with RNAP and their in vitro response to ppGpp.

In this report, we present a quantitative study of the effect of ppGpp on transcription initiation from the *rplJ* and *rpsA* P1 promoters. We demonstrate that the nature and extent of inhibition by ppGpp are influenced by the different affinities of the two promoters for RNAP. These findings are substantiated by measurement of the kinetic and

equilibrium constants of the individual steps leading to open complex formation, indicating that the mechanism of inhibition by ppGpp shows promoter-specific variations.

2. Materials and methods

2.1. DNA fragments and RNA polymerase

The plasmids pRLG597 [26], pJLO2, pSP261 and pKB252 [9] carrying the *rrnB*P1, *rplJ, rpsA* and *lacUV5* promoters, respectively, were digested as described earlier. The reactions were loaded on a 5% polyacrylamide gel, run in 1X TBE (90 mM Tris–Borate, 1 mM EDTA); the promoter-bearing fragments were excised from the gel, eluted and end-filled, according to standard methods [27]. For the filter-binding experiments, α-[^{32}P]dATP (3000 Ci mmol^{-1}), was included in the end-filling reactions. All fragments were further purified by passage through an Elutip-d mini-column (Schleicher and Schuell, GmbH) and concentrations were determined spectrophotometrically. RNA polymerase was purified according to Burgess and Jendrisiak [28], with an additional step of purification by Mono-Q chromatography [29]. The purified enzyme was $>95\%$ pure as judged by Coomassie-staining of SDS-polyacrylamide gels. Densitometric scanning showed the σ^{70} content of the holoenzyme to be at least 80%. The percentage of RNAP molecules active in binding to linear DNA fragments was determined in a gel-retardation assay, by titrating the holoenzyme (final concentration 2–200 nM) against an end-labelled T7-A1 promoter fragment (1 nM), at 30°C, in the standard transcription buffer (see below) containing 50 mM KCl. Following a 10-s heparin (50 μg ml^{-1}) challenge, free and bound DNA were separated on a 6% polyacrylamide gel in 1 × TBE, running at a constant 12 V cm^{-1}. The autoradiogram was scanned on a Molecular Dynamics scanning densitometer, the fraction of bound DNA was plotted against RNAP concentration, [RNAP]. From the value of [RNAP] required to give a plateau-value of bound DNA, the percentage of active molecules was determined to be $\sim 60\%$, assuming 1:1 stoichiometry of binding (data not shown). All measurements pertaining to association and dissociation reactions, involve *active* RNAP concentrations; for in vitro transcription assays, the *total* RNAP concentration is referred to.

2.2. In vitro transcription assays

Single-round, heparin-resistant, run-off transcription assays were carried out as described earlier [23]. RNAP concentrations were 10-fold higher than promoter concentrations to ensure saturation of all promoter sites. Promoter fragment (0.2 pmol) and 2 pmol of RNAP were incubated at 37°C in a transcription buffer containing 40 mM of Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 100 μg ml^{-1} BSA and 5% glycerol, in a reaction volume of 30 μl, for 15 min. When present, ppGpp at the indicated concentrations was preincubated with RNAP in the above buffer for 10 min at 37°C, prior to the addition of DNA. For the experiments shown in Fig. 1, 50 mM K-glutamate was used, while the experiments shown in Fig. 2 were carried out in the presence of 50 mM KCl. The reactions were started by adding 10 μl of a pre-warmed heparin-substrate mix containing 100 μg ml^{-1} heparin, 320 μM ATP, CTP and GTP, 100 μM UTP and 5 μCi α-[^{32}P]UTP (3000 Ci mmol^{-1}), in the standard buffer described above. The reaction was allowed to proceed for 5 min and 20 μl of stop solution was added. For the purpose of quantitation, 3–5 μl of an end-labelled dsDNA fragment (act. > 100 cps) was added as a recovery marker and the entire solution was precipitated overnight at −20°C with 2.5 volumes of 100% ethanol. After a brief rinse with 70% ethanol, the samples were dried, dissolved in 20 μl of formamide loading dye, heated to 90°C for 5 min and rapidly cooled on ice. The samples were loaded onto an 8% denaturing polyacrylamide gel containing 8 M urea and run in 1X TBE at constant voltage. The undried gels were exposed to X-ray films with two intensifying screens for 24–36 h at −70°C. The autoradiograms were scanned on a Molecular Dynamics Scanning Densitometer; for each lane, transcript intensities were normalized to the intensity of the recovery marker in that lane. The extent of inhibition is

the difference between intensities without and with ppGpp, expressed as a percentage of the (−ppGpp) transcript intensity.

2.3. Nitrocellulose filter-binding assays

Nitrocellulose BA-45 (NC) and diethylaminoethyl NA-45 (DEAE) membranes (Schleicher and Schuell, GmbH) were treated as described previously [30] soaked in the standard filter-binding buffer [40 mM Tris–HCl (pH 8.0), 100 mM K-acetate, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 100 μg ml^{-1} BSA and 5% glycerol] for 30 min at 4°C, and were layered between the plates of a 96-well dot-blot apparatus (Bio-Rad Inc.). All samples were handled in triplicate with multi-channel pipettors; loading times were typically less than 10 s. The filtered samples were washed with 100 μl of wash buffer (identical to the binding buffer, except for the replacement of 50 mM K-acetate by 100 mM KCl), air-dried and autoradiographed. The non-specific retention of DNA on NC was less than 0.5% of the total input DNA and retention efficiency of the polymerase–promoter complexes investigated here, was 80%. Retention due to non-specific RNAP–DNA complexes at high RNAP concentrations were found to be less than 3% of the total input DNA, using a promoter-less 242-bp pBR322 fragment. Accordingly, correction for fractional retention (θ), defined as the ratio of counts on NC to total counts (NC + DEAE), was done as described previously [30].

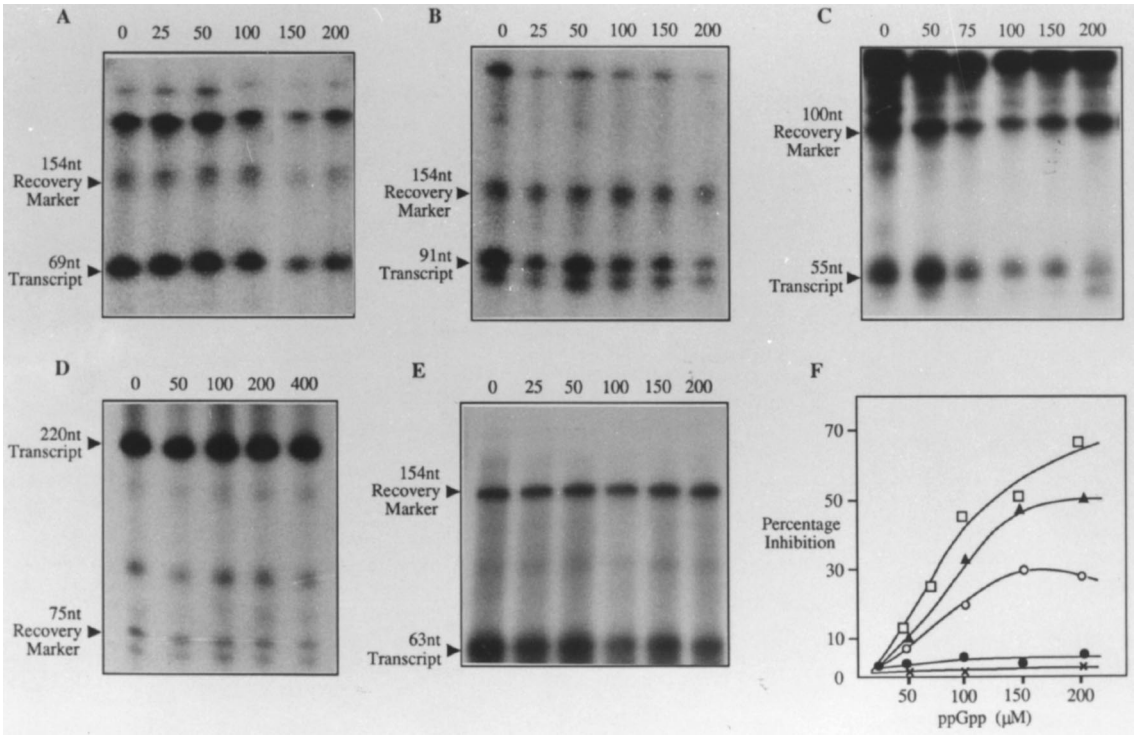
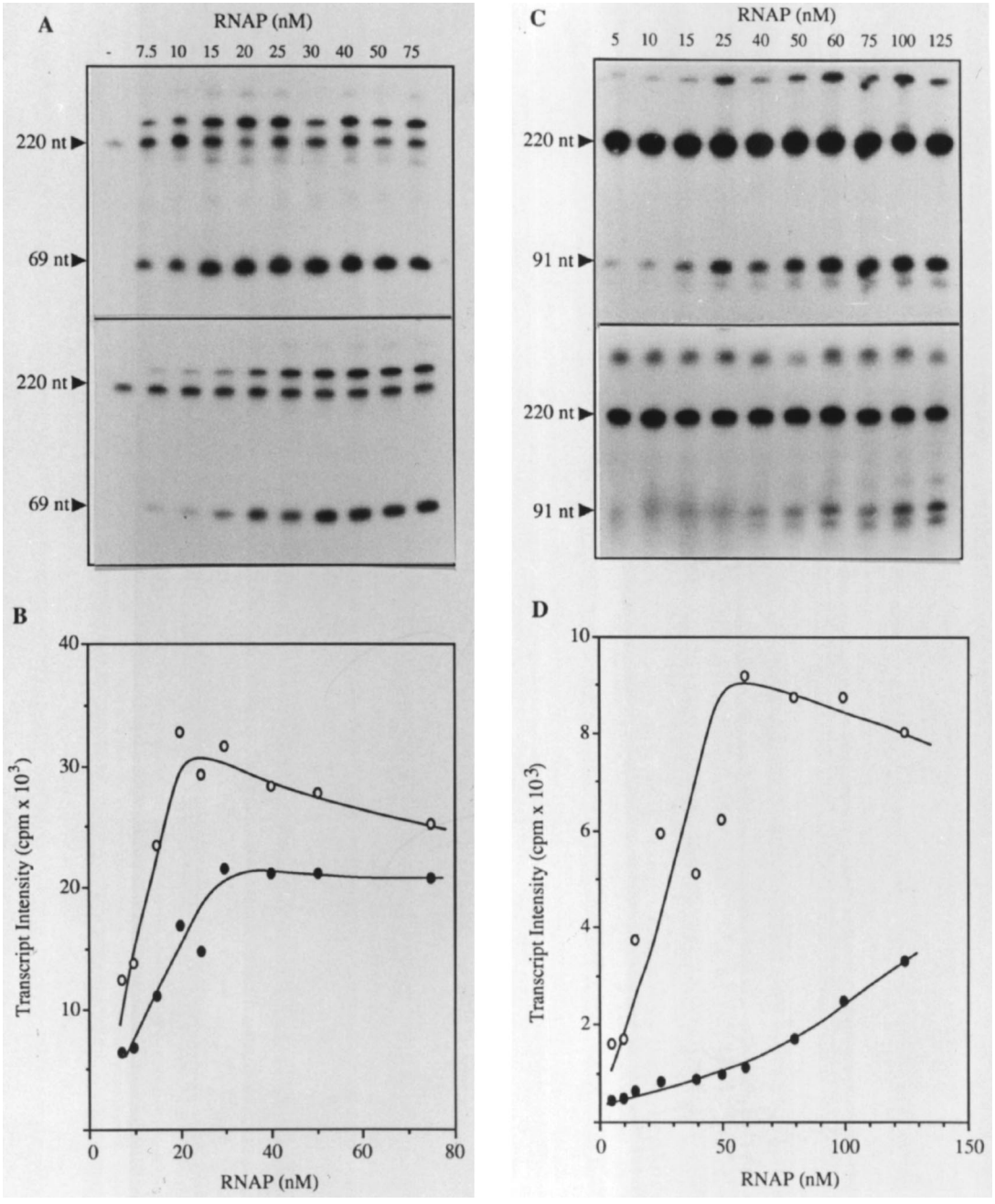


Fig. 1. In vitro transcription from rRNA, ribosomal protein and *lacUV5* promoters as a function of ppGpp concentration. (**A**) *rplJ*; (**B**) *rpsA* P1; (**C**) *rrnB* P1 (linear template); (**D**) *rrnB* P1 (supercoiled template); and (**E**) *lacUV5*. For calculation of the extent of inhibition of transcription (**F**), transcript intensities were normalized with respect to that of the recovery marker in the corresponding lanes; percentage inhibition was calculated relative to the transcipt intensities obtained without ppGpp. The symbols (x), (□), (●), (▲), and (○), correspond to the *rrnB* P1 (supercoiled), *rrnB* P1 (linear), *lacUV5*, *rplJ* and *rpsA* P1 templates, respectively.

2.4. Measurement of open complex formation and construction of τ-plots

Open complexes at the *rplJ, rpsA* P1 and *lacUV5* promoters were formed at 37°C in the standard binding buffer, by incubating labelled DNA (final concentration 0.2–0.5 nM) with various concentrations of RNAP holoenzyme in 200-μl reactions. When present, ppGpp was at a concentration of 100 μM; RNAP was preincubated with



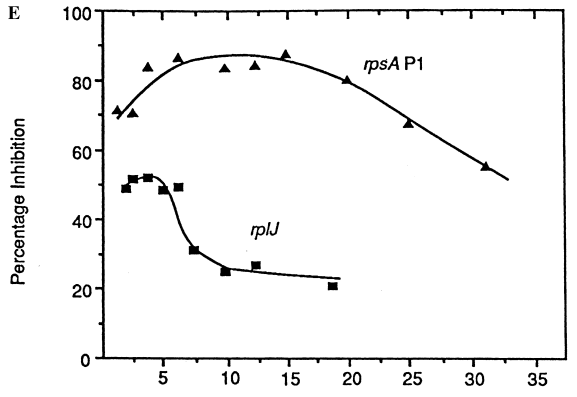


Fig. 2. Effect of ppGpp on in vitro transcription from *rplJ* and *rpsA* P1 promoters as a function of RNAP concentration. (**A**) Transcription at *rplJ* without (upper panel) and with (lower panel) 100 μM ppGpp; sizes of the recovery marker and transcript are 220 nt and 69 nt, respectively. (**B**) Normalized *rplJ* transcript intensities vs. RNAP concentration without (○) and with ppGpp (●). (**C**) Transcription at *rpsA* P1 without (upper panel) and with (lower panel) 100 μM ppGpp; sizes of the recovery marker and transcript are 220 nt and 91 nt, respectively. (**D**) Normalized *rpsA* P1 transcript intensities vs. RNAP concentration without (○) and with ppGpp (●). (**E**) Percentage inhibition of transcripts (calculated with respect to the reactions without ppGpp) vs. ratio of RNAP to DNA concentrations at *rplJ* (■) and *rpsA* P1 (▲) promoters.

ppGpp for 10 min prior to the addition of DNA. In order to measure the association kinetics, 30-μl aliquots were withdrawn at various time-points, mixed with 10 μl of pre-warmed heparin (final concentration 25 μg ml^{-1}) for 10 s, filtered and washed as described above. The triplicate values of θ at each time-point were averaged and the observed pseudo-first order rate constant of association (k_{obs}) was obtained from the slope of a plot of $\ln(1-\theta)$ against time. The $1/k_{obs}$ values at different RNAP concentrations, were plotted against 1/[RNAP] to yield τ-plots for the association reaction at each promoter.

Analysis of the association reaction was based on the two-step model of McClure [31]. This can be written as:

$$R + P \overset{K_B}{\rightleftharpoons} RP_C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

where R and P denote concentrations of RNAP and promoter, RP_c and RP_o are the closed and open complexes, respectively and $K_B = (k_1/k_{-1})$. Assuming pseudo-first order conditions ($R \gg P$), irreversible RP_o formation ($k_2 \gg k_{-2}$) and rapid equilibrium formation of RP_c ($k_{-1} \gg k_2$), the average time required for open complex formation (τ_{obs}) is given by:

$$\tau_{obs} = 1/k_{obs} = K_B k_2 [R]^{-1} + (k_2)^{-1}$$

The reciprocal slope and intercept of a plot of τ_{obs} vs. $[R]^{-1}$ (τ-plot), corresponds to the second-order association rate constant (k_a) and isomerisation constant (k_2), respectively, such that $k_a = K_B k_2$.

2.5. Test of the rapid equilibrium assumption

rplJ / rpsA P1 promoter fragments (0.03 fmol) were incubated with a fivefold excess RNAP at 20°C in 60-μl reactions containing 40 mM HEPES-KOH (pH 7.5), 50 mM K-acetate, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 μg ml^{-1} BSA and 5% glycerol. At fixed times, the reaction mix was rapidly diluted into 120 μl of 75 μg ml^{-1} heparin (in the above buffer) at 37°C, 30-μl aliquots from this solution were filtered and washed at intervals of 20–60 s. A 300-μl control reaction was set up to monitor RP_o formation at 20°C. Aliquots (30-μl) were withdrawn at various times, mixed with 10 μl of heparin pre-cooled to 20°C (final concentration 25 μg ml^{-1}) and the solutions were filtered. The bound complexes were washed with 100 μl of the buffer described above at 20°C. The average θ values from three independent control and test experiments at each promoter were used to obtain a plot of $\ln(1-\theta)$ against time.

2.6. Measurement of the dissociation reaction

Binary complexes were formed at 37°C by incubating labelled DNA (final concentration 0.2–0.5 nM) with a 10-fold molar excess of active RNAP holoenzyme for 10 min in 300-μl reactions in the standard filter binding buffer. When present, ppGpp (final concentration 100 μM) was preincubated with RNAP prior to DNA addition.

After adding 10 μl of pre-warmed heparin (final concentration 10 μg ml^{-1}), the dissociation reaction was followed by withdrawing 30-μl aliqouts from each reaction at different times; these were filtered and washed as described above. Separate control reactions with each promoter were carried out to follow the dissociation of open complexes in the absence of heparin. Average values of $\ln(\theta)$ from three independent measurements were plotted as a function of time and the slopes of the unweighted least-squares lines, gave the first order dissociation constant, k_d.

3. Results

3.1. Comparison of inhibitory effects of ppGpp

The inhibition of in vitro single-round transcription from the *rplJ* and *rpsA* P1 promoters has been previously studied using a mixed-template assay [9]. This assay offers a novel approach to mimic in vivo conditions of promoter selectivity, but it may not accurately reflect the in vitro sensitivity of promoters to ppGpp, in the absence of other competing templates. We therefore tested the response of the *rplJ* and *rpsA* P1 promoters individually, to increasing ppGpp concentrations, and compared the extents of inhibition with those of the stringent *rrnB* P1 and non-stringent *lacUV5* promoters, under the same reaction conditions.

In vitro, single-round transcription from these templates as a function of ppGpp concentration, is shown in Fig. 1. The inhibition of the 69 nt *rplJ* transcripts (Fig. 1A) was weak, with a maximum of 30% inhibition at 150 μM of ppGpp (Fig. 1F). With the *rpsA* P1 promoter (Fig. 1B), the extent of inhibition was higher compared with *rplJ* at all concentrations of ppGpp, with an almost twofold difference evident at 200 μM ppGpp (Fig. 1F). Saturation of inhibition at both promoters, occurs in the range 100–200 μM ppGpp. We also observed that when transcriptions were carried out with lower enzyme concentrations and 50 mM KCl, the inhibition at both promoters was higher; this aspect is addressed in Section 3.2.

The extent of inhibition of the 55 nt run-off transcript from the linear *rrnB* P1 fragment (Fig. 1C), was comparable with that of *rpsA* P1 and was twofold larger than that of *rplJ* in the range 100–200 μM. We observed no inhibition of the 220 nt terminated-transcript from the super-coiled *rrnB* P1 template even with 400 μM of ppGpp (Fig. 1D). At both *rrnB* templates, open complexes were formed in the absence of ATP/CTP and to enhance transcription from the linear *rrnB* P1 template under these conditions, 50 mM K-glutamate was used. In the presence of these nucleotides, the inhibitory effect of ppGpp was very weak and was observed only at linear *rrnB* P1 (data not shown). Our results agree with the observation that inhibition by ppGpp at *rrnB* P1 occurs only when supercoiling or addition of ATP/CTP have not driven open complex formation [10]. However, we must mention here that such dependence of transcription rate on template supercoiling could be noticed only in vitro, as in vivo, where maximum effect of ppGpp is seen, all the *rrnB* promoters are in a supercoiled state and our results do not represent the correct situation at least with respect to transcription at rRNA promoters. As expected, synthesis of the 63 nt transcript from the *lacUV5* promoter (Fig. 1E), was only marginally affected by ppGpp.

3.2. Saturation characteristics of the rplJ and rpsA P1 promoters

We then examined in vitro transcription at the two ribosomal protein promoters as a function of RNAP concentration ([RNAP]). As the percentage of active RNAP molecules was $\sim$60%, a range of concentrations was chosen to ensure at least a 10-fold molar excess of active RNAP over DNA. In order to compare the effects of ppGpp, we consider percentage inhibition at the two promoters, as a function of the ratio of RNAP to DNA concentrations ([RNAP]/[DNA]).

In the absence of ppGpp, yield of the 69 nt *rplJ* transcript saturates with 15–40 nM RNAP (Fig. 2A, upper panel), and subsequently declines when [RNAP] exceeds 40 nM (Fig. 2B). Inhibition by ppGpp is evident at all enzyme concentrations (Fig. 2A, lower panel) and transcript yields saturate between 30 and 50 nM RNAP (Fig. 2B). At [RNAP]/[DNA] $\leq$ 5, the extent of inhibition is $\sim$50% and rapidly declines to $\sim$20% when

[RNAP]/[DNA] exceeds 10 (Fig. 2E). From the observation that at high [RNAP], inhibition by ppGpp is only partially relieved, we infer that a step subsequent to promoter binding is affected by ppGpp. These experiments cannot distinguish whether the transition from closed to open complex, promoter clearance or even elongation are affected.

The *rpsA* P1 promoter shows three to fourfold weaker transcription activity than *rplJ*, requiring a 15-fold molar excess of enzyme for maximal in vitro activity (Fig. 2C, upper panel). Transcript yields in the absence of ppGpp, saturate with 50–70 nM RNAP (Fig. 2D) and decline only when enzyme concentrations exceed 80 nM. In the presence of ppGpp (Fig. 2C, lower panel), inhibition at all enzyme concentrations is much stronger than at *rplJ*. Transcript yields do not saturate, but show a steep increase when [RNAP] exceeds 60 nM (Fig. 2D). The corresponding extents of inhibition are 80–85% when [RNAP]/[DNA] is between 5 and 20 (Fig. 2E), and $< 70\%$ when [RNAP]/[DNA] exceeds 25. The requirement of high enzyme concentrations ([RNAP] > 60 nM) for significant transcription activity and the high extent of inhibition ($\sim 80\%$) over a 10-fold range of [RNAP]/[DNA], strongly indicates that ppGpp inhibits RNAP binding to the *rpsA* P1 promoter. However, we cannot rule out the inhibition of the subsequent steps of transcription by ppGpp.

For the transcription reactions carried out with 50 mM KCl (Fig. 2A,C), the extents of inhibition at *rplJ* and *rpsA* P1 are approx. 30 and 80%, respectively, at [RNAP]/[DNA] = 10 (Fig. 2E). In contrast, the extents of inhibition of transcription when 50 mM K-glutamate is used (Fig. 1F), are $\sim 20\%$ at *rplJ*, and $\sim 35\%$ at *rpsA* P1. We attribute the lesser inhibition observed in Fig. 1F to the stabilizing influence of K-glutamate on protein–nucleic acid interactions [32].

3.3. Measurement of the rate constants of open complex formation

In order to verify whether ppGpp shows differential effects on the binding of RNAP and the conversion of RP_c to RP_o, we measured the rate constants of open complex (RP_o) formation at the *rplJ* and *rpsA* P1 promoters in the absence and presence of ppGpp. To assess the significance of any changes observed in the presence of ppGpp, we have carried out similar measurements with the non-stringent *lacUV5* promoter. Based on the two-step model of McClure, we interpret the experimentally determined second-order rate constant (k_a) in terms of the elementary equilibrium (K_B) and isomerisation (k_2) constants. This is possible only if the reaction proceeds by the rapid equilibrium mechanism, where $k_{-1} \gg k_2$. If $k_2 \gg k_{-1}$, the sequential mechanism applies and $k_a = k_1$ [31].

To determine which of these two limiting mechanisms describe open complex formation at *rplJ* and *rpsA*P1 promoters. (The rapid equilibrium formation of RP_c at *lacUV5* is assumed [33]), we assayed the conversion of closed complexes (formed by incubating polymerase and promoter fragments at 20°C) to open complexes, following a temperature jump to 37°C in the presence of 25 μg ml^{-1} heparin (see Section 2). At both promoters, the extent of RP_o formation [described by the decrease in $(1-\theta)$], was essentially invariant (Fig. 3), indicating that negligible conversion of RP_c to RP_o took place in presence of heparin. Since heparin acts only on free RNAP in the time-scale of this experiment (see below), we infer that the heparin sensitivity of RP_c must result from the rapid conversion of RP_c to free RNAP, rather than RP_o, leading to the condition that $k_{-1} \gg k_2$.

Fig. 4A shows the τ-plot for the *rplJ*–polymerase interaction at 37°C. The low slope of the least-squares line in the absence of ppGpp and the large k_a value (6.7×10^7 M^{-1} s^{-1}) is consistent with our results that show a rapid saturation of single-round transcription (Fig. 2A). In comparison, the τ-plot for the *lacUV5* promoter (Fig. 4C) yields higher k_a (9.6×10^7 M^{-1} s^{-1}) and k_2 (11.3×10^{-2} s^{-1}) values. In the presence of ppGpp a twofold decrease in both k_a and k_2 is observed at *rplJ*, but there is no significant change in K_B (Table 1). For the *lacUV5* promoter, there is no significant effect of ppGpp on either k_a or k_2. We conclude that the rate-limiting step in open complex formation at *rplJ* in the presence of ppGpp, is the conversion of RP_c to RP_o. Our

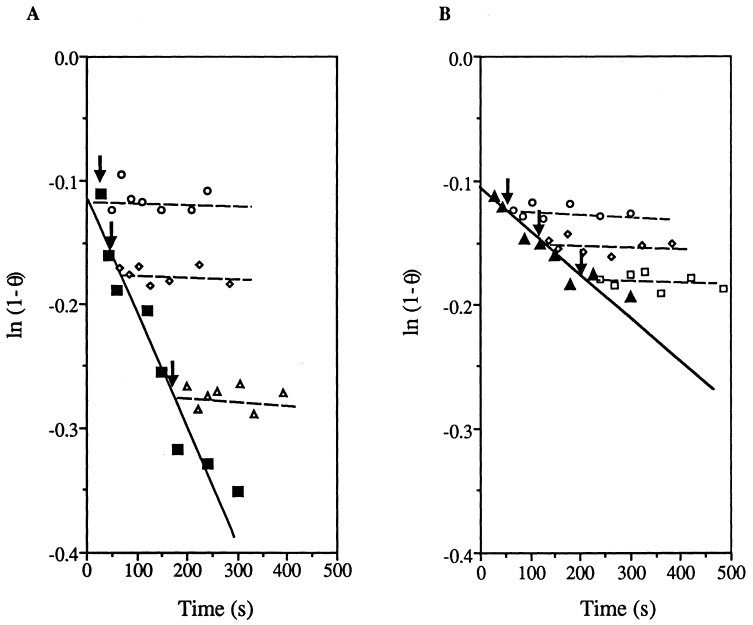


Fig. 3. Test of the rapid equilibrium formation of closed complexes at the *rplJ* and *rpsA* P1 promoters. The time-courses of open complex formation at 20°C for control reactions are shown for **(A)** *rplJ* (■) and **(B)** *rpsA* P1 (▲) promoters. The symbols (○), (◇) and (Δ) for *rplJ*, (□) for *rpsAP1* indicate the extent of open complex formation of three separate reactions at each promoter that were treated with heparin at 37°C, at the time points indicated by the arrows (see Section 2). The dashed lines represent the best fits through the data points.

experiments do not allow interpretation of this process in terms of two-intermediate mechanisms [34] where a conformational change in RNAP after promoter-binding and subsequent promoter-melting can be differentiated.

With the *rpsA* P1 promoter, the steeper slopes of the least-squares lines of the τ-plot (Fig. 4B), indicate that open complex formation at this promoter is more sensitive to variations in RNAP concentration. The threefold lower k_a values in the absence of ppGpp, indicate a weaker extent of open complex formation with respect to *rplJ*. The effect of ppGpp addition is a further threefold reduction in k_a (0.6×10^7 M^{-1} s^{-1}) and a relatively smaller decrease in k_2. The resulting twofold decrease in K_B ($0.65–0.22 \times 10^9$ M^{-1}) is significant compared with the smaller changes at the *rplJ* and *lacUV5* promoters (Table 1), and we consider that the formation of RP_c is rate-limiting at this promoter. These results, however, do not satisfactorily account for the high level of inhibition (80–85%) seen in single-round transcription.

Table 1
Equilibrium and kinetic parameters of open complex formation at the *rplJ*, *rpsA* P1 and *lacUV5* promoters

	ppGpp (100 μM)	k_a (-10^{-7} M^{-1} s^{-1})	k_2 ($\times 10^2$ s^{-1})	$K_B = k_q = k_a/k_2$ ($\times 10^{-9}$ M^{-1})	k_d ($\times 10^5$ s^{-1})
	−	6.7 ± 0.8	5.5 ± 0.9	1.22 ± 0.08	4.8 ± 1.3
rplJ	+	3.2 ± 0.4	2.7 ± 0.7	1.18 ± 0.17	9.8 ± 1.7
	−	2.0 ± 0.9	3.1 ± 1.3	0.65 ± 0.03	4.9 ± 0.6
rpsA P1	+	0.6 ± 0.2	2.7 ± 1.1	0.22 ± 0.02	6.7 ± 1.8
	−	9.6 ± 1.9	11.3 ± 2.4	0.85 ± 0.01	2.6 ± 0.3
lacUV5	+	7.6 ± 1.4	8.2 ± 1.7	0.93 ± 0.02	3.1 ± 0.5

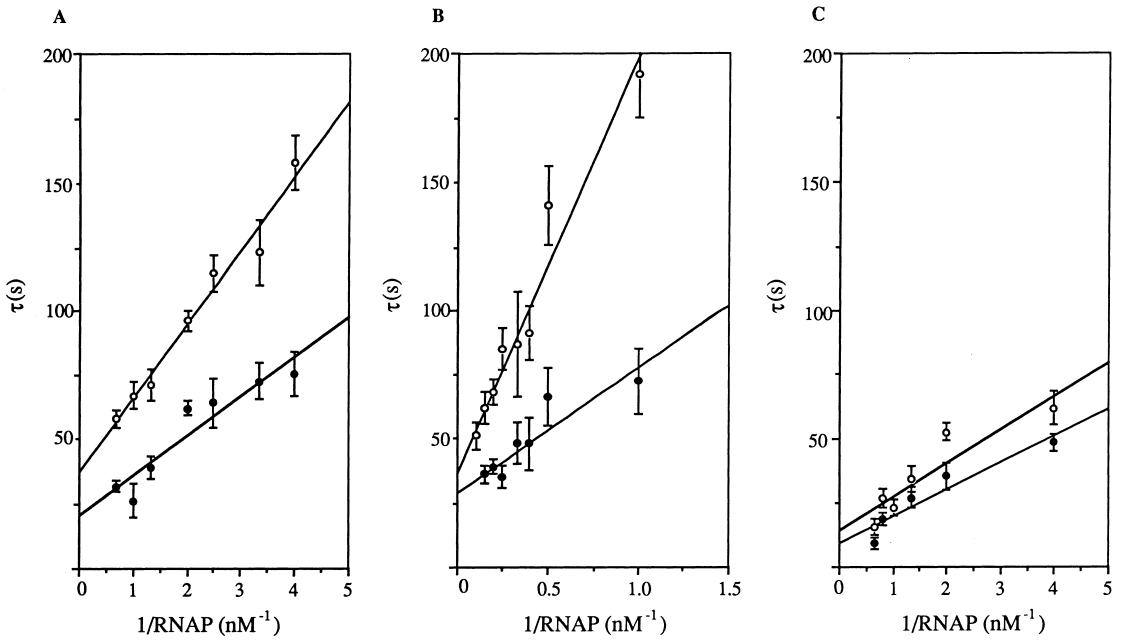


Fig. 4. τ-plots for the association reaction between (**A**) 0.2 nM *rplJ*; (**B**) 0.5 nM *rpsA* P1; and (**C**) 0.2 nM *lacUV5* promoters. The values of τ ($1/k_{obs}$), are the average of four independent measurements at the indicated RNAP concentrations. Error bars in each plot are $\pm$ 1 S.D. of the data. Slopes and intercepts are calculated from the unweighted least-squares fit to the data. In all cases RNAP concentrations reflect the population of molecules active in binding to linear DNA templates. -○-(−ppGpp); -●-(+ppGpp).

We therefore conclude that additional effects of ppGpp on the rates of promoter clearance or elongation/pausing contribute to the inhibition of in vitro transcription from *rpsA* P1.

The values of k_a obtained with the *lacUV5* promoter ($\approx 9.0 \times 10^7$ M^{-1} s^{-1}), are approximately five- to 10-fold higher compared to previous estimates obtained with the abortive initiation assay [35] and by nitrocellulose filter-binding [33]. We attribute these differences to the stabilizing effect of acetate ions on open complex formation [32]. We found that the use of 50 mM K-acetate vastly enhanced the efficiency of filter-retention, resulting in greater precision in estimating θ.

3.4. Estimation of the dissociation rate constant (k_d)

In order to determine if the instability of the open complex as described previously [9] contributes to the inhibition of transcription, we followed the irreversible dissociation of pre-formed open complexes in the presence of heparin. At all three promoters, the long lifetimes of the complexes (Fig. 5) indicates that the conversion of RP_c to RP_o is essentially irreversible on the timescale of RP_o formation, and supports the assumption that $k_2 \gg k_{-2}$. We found that by varying heparin concentrations in the range 25–100 μg ml^{-1}, there was no significant effect on the rates of dissociation (data not shown) and we conclude that the observed (k_{obs}) and intrinsic (k_d) dissociation constants are identical [36]. In conjunction with the assumption of rapid equilibrium between RP_c and free RNAP, the dissociation reaction can be written as:

$$RP_o \xrightarrow{k_d} RP_c \longrightarrow R + P$$

In the absence of ppGpp, k_d for the two ribosomal protein promoters are similar, and are approximately twice as large as the value obtained

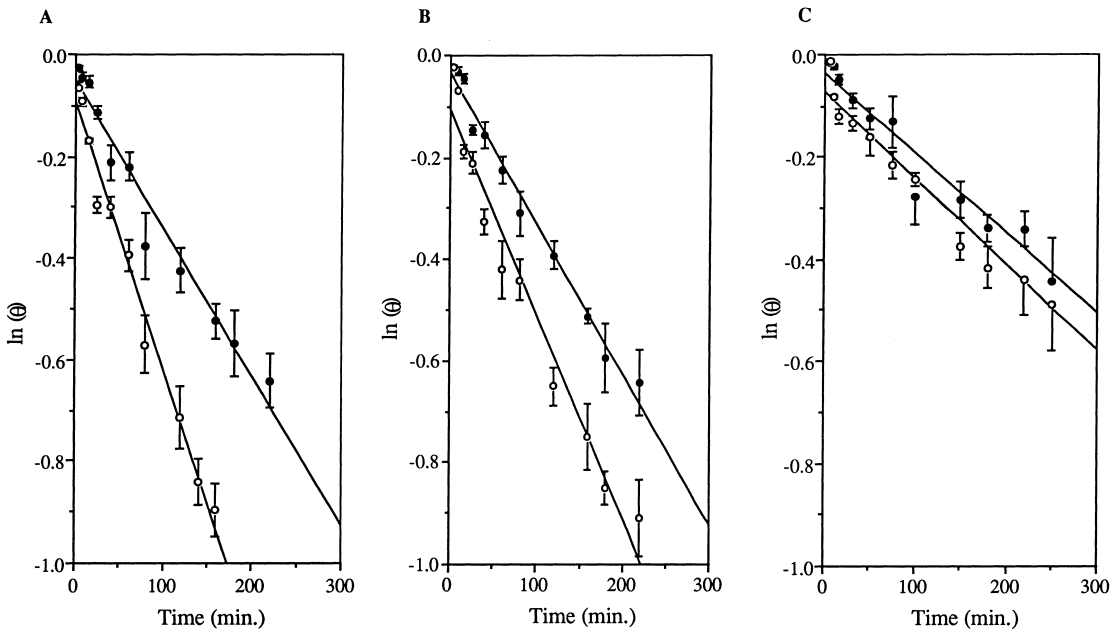


Fig. 5. Measurement of the dissociation reaction at (**A**) *rplJ*; (**B**) *rpsA* P1; and (**C**) *lacUV5* promoters. DNA concentrations were identical to those in Fig. 4. The values for the fractional retention (θ) of DNA are the average of five separate measurements; error bars indicate $\pm$ 1 S.D. of the data. The fit to the data represents the unweighted linear least-squares line.

at *lacUV5* (Table 1). In the presence of ppGpp, a significant twofold increase in k_d is seen at the *rplJ* promoter, while the changes at *rpsA* P1 and *lacUV5* are less significant. This result indicates that ppGpp affects the stability of the open complex at *rplJ*, and strengthens our conclusion that the transition from RP_c to RP_o is rate-limiting at this promoter.

4. Discussion

In *Escherichia coli*, the inhibition of transcription by ppGpp is influenced by the presence of specific promoter sequences, physiological variables [13,18] and by the properties of transcription complexes at individual promoters [10]. Differences in the extents of inhibition by ppGpp highlight the contrasting responses of in vitro transcription from the *rplJ* and *rpsA* P1 promoters to RNAP concentration. At high [RNAP], transcript yields at both promoters in the absence of ppGpp, decrease by $\sim$ 10% of the saturation value (Fig. 2B,D). Two proposed mechanisms account for the reduced extent of open complex formation [37] and altered distribution and yields of abortive products [38] with high RNAP concentrations in vitro. Either of these mechanisms could explain the reduced transcript yields at *rplJ* and *rpsA* P1, but the experiments described here cannot distinguish between them. We wish to emphasise the differences between ppGpp-inhibited transcription from the two promoters (Fig. 2B,D). At *rpsA* P1, a 15-fold excess of RNAP is required for significant transcription activity, while the same RNAP concentration leads to saturation of transcript yields at *rplJ*. We interpret this result as being due to a differential effect of ppGpp-binding on the affinities of RNAP for the two promoters; ppGpp exerts a stronger inhibitory effect on promoter-binding at *rpsA* P1, than at *rplJ*.

The macroscopic rate constants obtained from the two-step formalism of McClure suffice for a comparative account of the response the *rplJ* and *rpsA* P1 promoters to ppGpp. The two-step model has been used to measure the effects of various

mutations on open complex formation at λP_R [39,40] and at Class II CAP-dependent promoters [41]. Although we cannot rule out effects of non-specific binding at high RNAP concentrations, we believe they will have little effect considering the range of RNAP concentrations used in this study. The high k_a of the *rplJ* promoter (relative to *lacUV5*) is not unusual, considering that it drives a high level of expression in vivo [21]. Considering the negligible change in K_B (Table 1) and the twofold changes in k_2 and k_d with respect to the weaker effects at *rpsA* P1 and *lacUV5*, we propose that ppGpp enhances the decay of RP_o at *rplJ* but does not significantly affect the closed complex RP_c. The rapid saturation characteristics of single-round transcription in the presence of ppGpp (Fig. 2B), further support our contention that RP_c formation is not affected. We envisage that in the presence of NTPs, the RP_o species can rapidly convert into a stable ternary complex, as shown for the *rrnB* P2 promoter [42]. In contrast, the twofold decrease in K_B at *rpsA* P1 (Table 1) and the requirement of a 20-fold molar excess of RNAP over DNA for significant activity (Fig. 2D), indicate that ppGpp predominantly affects the binding of RNAP to this promoter. The twofold lower K_B values in the absence of ppGpp compared with *rplJ*, reflects the intrinsically weaker extent of RP_c formation. It is possible that low salt conditions and presence of initiating NTPs may increase the rate of RP_c formation, as demonstrated for the *rrnB* P1 promoter [43]. We have shown substantially weaker inhibition of single-round transcription at *rpsA* P1 by ppGpp, when reactions are carried out in the presence of K-glutamate rather than KCl (cf. Fig. 1F and Fig. 2E). In the subsequent paper we show that in both cases, a large fraction of the total holoenzyme is dissociated from the binary transcription complex and that the results of our kinetic studies can be incorporated into a model to account for the differences in the extents of formation of various intermediate binary species.

The precise mechanism of ppGpp-mediated inhibition remains a long-standing problem in the stringent regulation of transcription initiation. We have demonstrated a differential in vitro effect of ppGpp at two poorly characterized stringent ribosomal protein promoters. Our findings strengthen the possibility that diverse, promoter-specific mechanisms are responsible for the stringent control of transcription initiation, as opposed to a unitary mechanism applicable to all stringently regulated promoters.

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