ppGpp regulates the binding of two RNA polymerase molecules to the *tyrT* promoter

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Abstract

Bacterial promoters differ in the number of RNA polymerase molecules that bind to form a filterable polymerase-promoter complex. We show that two holoenzyme molecules interact with the *tyrT* promoter, probably as a dimer. This interaction is inhibited by ppGpp. By contrast a single holoenzyme monomer suffices for complex formation at the *lacUV5* promoter. We propose that *In vivo* promoter selection by monomeric and dimeric forms of the enzyme could coordinate the synthesis of stable RNA with that of mRNA and could also account in part for the switch in transcriptional selectivity during the stringent response.

Introduction

A necessary step in the synthesis of a bacterial RNA molecule is the formation of a productive RNA polymerase-promoter complex, generally termed the 'rapid start' or 'open' complex (1). Maximal initiation at many mRNA promoters, such as *lacUV5*, is favoured *in vitro* by high ionic strengths (2). By contrast promoters directing stable RNA synthesis initiate most efficiently at low ionic strength ($\mu \leq 0.05$) (3 - 5). This switch in transcriptional selectivity parallels the aggregation of two RNA polymerase holoenzyme molecules to a dimer at low salt (6,7). We show here that the promoter directing the transcription of the *tyrT* gene, which encodes a major tRNA^{Tyr} species in *E. coli*, requires the interaction of approximately two holoenzyme monomers for the formation of a filterable polymerase-promoter complex while the *lac UV5* promoter requires only a single holoenzyme molecule for efficient retention in a filter binding assay. We propose that the dimerisation of polymerase monomers is required for the formation of open complexes at promoters with particular structural features. Promoter selection by RNA polymerase would thus be dependent on the position of the equilibrium between polymerase monomers and dimers.

In vitro, and also probably in vivo, promoter selection is regulated by guanosine 5' diphosphate 3' diphosphate (ppGpp), a nucleotide which accumulates to millimolar levels in rel⁺ bacteria starved for a required aminoacid (8,9). We show here that the nucleotide affects the binding characteristics of RNA polymerase to the wild type and to a mutant tyrT promoter and also to the lac UV5 promoter in a manner similar to increasing ionic strength. We propose that one way in which ppGpp alters promoter selectivity is to alter the equilibrium between polymerase monomers and dimers in favour of monomers.

Materials and Methods

Materials

E. coli RNA polymerase holoenzyme was prepared by the method of Burgess and Jendrisak (10) as far as the elution of the enzyme from the Polymin P precipitate. The Polymin P eluate was then adsorbed to a DE-52 column and eluted with a linear gradient of 0.1 to 0.5M KCl in TGED (10). The enzyme activity was further purified by chromatography on a Biogel A 1.5m column followed by adsorption to a heparin-Sepharose column. This latter column was developed with a 0.2 to 1.0M KCl linear gradient with TGED as buffer. Holoenzyme containing 1 mole σ subunit/2 moles α subunit was then prepared by the method of Gonzalez et al. (11).

The 207 bp restriction fragments containing the SSU1 and SSU2 sequences were prepared from pRD69 and pRD3 respectively (12,13) and the 205 bp restriction fragment containing the lac UV5 promoter from pOP1 (14). The plasmids were cleaved with Eco R1 and the resulting fragments separated by electrophoresis on a 10% polyacrylamide gel. All fragments were located by staining the gel briefly with ethidium bromide, eluted, and precipitated with ethanol. This precipitate was dissolved in 0.01M Tris HCl pH 7.9, 0.0001M EDTA, 0.1M KCl.

Eco R1 was the gift of Dr. J. Karn, Hha 1 and ppGpp were obtained from Biolabs Inc. and P.L. Biochemicals respectively.

Preparation of ³²P labelled restriction fragments.

Restriction fragments were labelled at their 3' termini by reverse transcriptase. Reaction mixtures (43 μ l) contained 0.1M Tris HCl pH 7.9, 0.01M MgCl₂, 0.0025M 2-mercaptoethanol, 0.14 pmoles DNA fragment 25 μ M each dTTP, dCTP and dGTP, 20 μ Ci α ³²P dATP (specific activity $\sim 4 \times 10^5$ curies/mole). The reaction was incubated at 37° for 2 hr and terminated by heating at 60° for 5'. The specific activity of fragments labelled in

this way was in the range 3×10^4 - 1.5×10^5 cpm/ μ g. Restriction fragments labelled by this method bound to RNA polymerase with similar characteristics to fragments which had not been treated with reverse transcriptase. We note that restriction fragments labelled using Klenow DNA polymerase bound preferentially to RNA polymerase compared to untreated fragments, as judged by the failure of the latter to compete the binding of labelled fragments.

Binding assay

Reaction mixtures (25 μ l) contained 0.08M Tris HCl pH7.9, 0.01M $MgCl_2$, 0.0002M dithiothreitol, 2.8×10^{-10} M DNA fragment with KCl and RNA polymerase concentrations as indicated. Reactions were started by the addition of RNA polymerase and continued for 15' at 22 $^{\circ}$ unless otherwise stated. The reaction mixture was then diluted with 5 ml washing buffer (0.04M Tris HCl pH 7.9, 0.01M $MgCl_2$ and 0.05M KCl) at 0 $^{\circ}$, mixed and filtered immediately through 2.5 cm HA 0.45 μ m millipore filters presoaked in the same buffer. The filters were washed with 10 ml cold washing buffer, dried and counted.

Results

Two RNA polymerase molecules bind to the tyrT promoter

To study the interaction of RNA polymerase with the tyrT promoter a 207 bp DNA fragment (SSU1) was isolated from pRD69 DNA (12,13) and labelled with ^{32}P at both 3' ends. This promoter contains two Hha I cleavage sites, one centred between positions -24 and -23 and the other between positions -5 and -4 (15). To establish the specificity of polymerase binding we compared the binding of the intact and Hha I-cleaved fragments to RNA polymerase. At 114 fold molar excess of polymerase to DNA < 5% of the label in the cleaved fragment was retained on filters in contrast to approximately 60% of the intact fragment (Figure 1). Hha I cleavage of the SSU1 fragment yields two labelled products of unequal lengths (28 and 155 bps respectively), each labelled at only one end. If either of these fragments were preferentially bound by RNA polymerase the amount of total label retained in the filter binding assay would underestimate the retention of the preferentially bound fragment. In the extreme situation when only one of the two labelled fragments is bound by RNA polymerase retention of 5% of the total label would be equivalent to retention of 10% of the bound fragment. We can therefore conclude that the efficient retention of the SSU1 restriction fragment is not observed when

Figure 1. Specificity of RNA polymerase binding. o—o, intact SSU1 fragment; ●—● Hha I cleaved SSU1 fragment. KCl concentration was 0.075 M, 100% retention was 13,340 cpm. A background of 250 cpm is subtracted from all points.

the promoter region is cleaved at two sites. The cleaved fragment also fails to direct the synthesis of the 150 nucleotide RNA characteristic of initiation at the normal SSU1 promoter (Lamond, unpublished observations).

An unexpected feature of the binding of RNA polymerase to the intact fragment is the sigmoid dependence on RNA polymerase concentration (Figure 1) suggesting that the interaction of more than one RNA polymerase molecule might be necessary for the formation of a filterable complex at the SSU1 promoter. This data can be analysed by a double log plot of DNA retained against polymerase concentration. The slope, n , of this plot gives the number of molecular entities or functional units of RNA polymerase involved in the interaction. Analysis of the data in Fig. 1 in this way yields a value of $n = 1.8$ at intermediate polymerase concentrations where significant promoter dependent binding is observed.

This suggests that in this region the association of two RNA polymerase functional units is required for the formation of a filterable polymerase DNA complex.

The dimerisation of holoenzyme monomers is an example of an interaction involving two functional units of RNA polymerase (6,7). The equilibrium between the monomer and the dimer is dependent on ionic strength, the monomer being favoured by high ($\mu > 0.15$) and the dimer by low ionic strength ($\mu < 0.05$) (6,7). To test whether the value of n was also dependent on ionic strength, the binding characteristics of RNA polymerase to the SSU1 promoter were determined at 0.05M and 0.15M KCl. At the higher salt concentration the dependence of polymerase-promoter complex formation on enzyme concentration was sigmoid (Figure 2), yielding a value of $n = 1.7$. By contrast at 0.05M KCl the dependence was hyperbolic with $n = 0.9$. Thus at 0.15M KCl the data is consistent with two functional units of RNA polymerase being required for polymerase-promoter complex formation while at 0.05M KCl only a single functional unit is necessary. However the data does not distinguish whether the functional units required at low and high salt concentrations are the same or different. Using the same assay and method of analysis we observed that for polymerase-lacUV5 promoter complex formation $n = 0.44$ and 0.90 at low and high salt concentrations respectively (Figure 2). Thus, in contrast to the SSU1 promoter, only a single functional polymerase unit was necessary for filterable complex formation with this promoter at 0.15 M KCl.

By applying a Scatchard analysis to the binding data for which $n = 1$ the number of polymerase monomers required for binding to the SSU1 and lacUV5 promoters can be determined. Figure 3 shows, in agreement with a previous study (2), that at 0.15 M KCl approximately one holoenzyme monomer is involved in complex formation at the lacUV5 promoter. This result suggests that the functional unit of RNA polymerase at this salt concentration is a holoenzyme monomer, and further implies that the interaction of two monomers is required for complex formation at the SSU1 promoter. Similarly at 0.05 M KCl two holoenzyme molecules interact with the SSU1 promoter but in this case these two monomers comprise the functional unit of polymerase activity.

From the data of Figures 2 and 3 the apparent binding constants of RNA polymerase to the SSU1 promoter at 0.05 M KCl is $3.2 \times 10^8 \text{ M}^{-1}$ and to the lacUV5 promoter at 0.15 M KCl is $3.7 \times 10^8 \text{ M}^{-1}$. These values are similar to those observed for other promoters (for a discussion see

Figure 2. Effect of salt and 100 μM ppGpp on RNA polymerase binding to SSU1, lacUV5 and SSU2 promoters $\circ$ — $\circ$ 0.05 M KCl; $\square$ — $\square$, 0.15 M KCl; $\bullet$ — $\bullet$, 0.05 M KCl + 100 μM ppGpp. a) Plot of % retention against polymerase concentration. 100% retention was 32,191, 30,005 and 37,283 cpm for SSU1, lacUV5 and SSU2 respectively. b) Plot of log₁₀ (DNA bound) against log₁₀ (polymerase concentration).

Figure 3. Scatchard plot of data for polymerase binding to SSU1 and lacUV5 promoters. ○—○, lacUV5 at 0.15 M KCl, ●—●, SSU1 at 0.05 M KCl. Data plotted is DNA bound/enzyme concentration against DNA bound.

reference 16). The calculations assume that all polymerase molecules in the preparation are active and that the enzyme is functionally homogeneous.

ppGpp regulates polymerase binding to the tyrT promoter

The lacUV5 and tyrT promoters differ in their response to the RNA polymerase regulator ppGpp, which increases initiation at the lac promoter and decreases it at the tyrT promoter (17,18). The response to ppGpp is determined, at least in part, by a DNA sequence spanning positions -5 to +2 in the tyrT promoter (12). This region is altered in a chemically synthesised variant, SSU2 (13), of the tyrT promoter, the wild type sequence C G C C C G C T from -5 to +2 being changed to C G T T A G C T (the numbering is normalised to the consensus sequence for the -10 region). This change alters the in vitro response of transcription to ppGpp such that the SSU2 promoter responds in the opposite manner to SSU1 and in a similar manner to lacUV5 (12). By contrast, in the binding assay the SSU2 promoter behaves in a similar manner to SSU1, the values of n at

low and high salt concentration being approximately 0.8 and 1.8 respectively (Figure 2).

In addition to affecting transcriptional selectivity in vitro ppGpp also markedly reduces the optimal salt concentration for RNA synthesis (3,5,18). Since the binding characteristics of RNA polymerase to the SSU1, SSU2 and lacUV5 promoter containing DNA fragments are highly dependent on ionic strength (Figure 2) we determined the effect of 100 μ M ppGpp on polymerase-promoter complex formation. Figure 2 shows that at 0.05 M KCl ppGpp strongly inhibited polymerase binding to both the SSU1 and SSU2 promoters. At the same time the nucleotide altered the dependence of complex formation on enzyme concentration from a hyperbolic to a sigmoid mode such that the value of n was increased from 0.9 to 2.0. Under the same conditions 5' GDP did not influence fragment retention at concentrations up to 500 μ M (data not shown) showing that this effect was nucleotide specific.

This result shows that for the SSU2 promoter the effects of ppGpp on transcription and on RNA polymerase binding are clearly distinguishable. Similarly, although ppGpp stimulates transcription from the lacUV5 promoter (17), the nucleotide inhibited polymerase binding to this promoter at low enzyme concentrations but not at high (Figure 2). Again ppGpp doubled the value of n , increasing the control value of 0.44 to 1.0. Thus the values of n observed in the presence of 100 μ M ppGpp are very similar to those observed at high salt. In this respect at least, the effect of ppGpp on complex formation is equivalent to that of high ionic strength.

In the experiments reported here ppGpp alters the retention of polymerase-promoter complexes on nitrocellulose filters. This contrasts with the studies on the rrn promoters by Hamming et al., (19) who observed that ppGpp altered the nature but not the amount of the complexes retained. We note that our protocol involves a more extensive washing procedure which would be likely both to increase the selectivity of retention and to decrease the detection of the more rapidly dissociating complexes (20).

Polymerase-tyrT promoter complexes are heterogeneous

In this set of experiments we have measured the formation of a filterable RNA polymerase-DNA complex. Complex formation on the SSU1 fragment is strongly temperature dependent, approximately seven times more fragment being retained at 22° compared to 0° (data not shown). Once

formed ~ 60% of the complexes are dissociated by single stranded DNA while the remainder are resistant (Figure 4a). Single stranded DNA traps free RNA polymerase molecules and thus rapidly dissociating polymerase-promoter complexes are preferentially removed in its presence. The complexes formed at the SSU1 promoter thus clearly fall into two classes, one which dissociates rapidly, at least in the presence of single stranded DNA, and a second which is more stable. Comparison of Figures 1 and 4 suggests that the formation of both classes depends on the integrity of the SSU1 promoter.

The complexes sensitive to single stranded DNA are themselves heterogeneous. When 100 μ M ppGpp is added to polymerase-promoter complexes preformed at the SSU1 promoter for 15', about 25-30% of the complexes are dissociated (Figure 4a). Since complex dissociation induced by the simultaneous addition of ppGpp and single stranded DNA is indistinguishable from that induced by single stranded DNA alone (Figure 4b) the ppGpp sensitive complexes must be a subset of the single stranded DNA sensitive complexes. Thus at least three types of complex are formed between RNA polymerase and the SSU1 promoter. The minimal model for RNA chain initiation proposes that the rapid starting 'open' polymerase-promoter complex is preceded by the initial 'closed' complex (1). However the existence of an intermediate 'transition' complex has been postulated for open complex formation at certain regulated promoters (21). Indeed, the kinetics of open complex formation at the mutant λP_R promoter x3 are consistent with a more complicated mechanism (22). Previously the sensitivity of complexes to single stranded DNA has been used to distinguish open and closed complexes (19,23), as has the strong temperature dependence of open, but not of closed, complex formation. In our experiments the formation of both the single stranded DNA sensitive and the single stranded DNA resistant complexes is temperature dependent. Whether these observed complexes are on the direct pathway to open complex formation, or on non-productive side pathways remains to be established.

Discussion

We have shown that specific promoters differ in the number of RNA polymerase molecules required to form a filterable polymerase-promoter complex. In particular the SSU1 and SSU2 promoters need approximately twice as many polymerase molecules to form such a complex at both low and high ionic strength as does the lacUV5 promoter. Since a polymerase

Figure 4. Sensitivity of polymerase-SSU1 promoter complexes to single stranded DNA and ppGpp. Complexes were preformed by incubation at 22° for 15'. 50 µg/ml denatured calf thymus DNA was then added to half the reaction mixture and aliquots assayed for complex formation at selected times. a) o—o, control; □—□, + single stranded DNA; ●—●, + ppGpp. b) o—o, control; □—□, + single stranded DNA; ■—■, + single stranded DNA + ppGpp.

holoenzyme monomer suffices for complex formation at the lacUV5 promoter (ref. 2 and figure 3) the observations suggest that binding, although not necessarily initiation, at the SSU1 and SSU2 promoters requires two holoenzyme molecules.

Does this interaction of two enzyme monomers involve the direct binding of a polymerase dimer or the separate binding of two polymerase monomers to the SSU1 promoter? The functional unit of polymerase activity for binding to both the SSU1 and lacUV5 promoters comprises two polymerase monomers at low salt and a single monomer at high salt (Figure 2 and 3), and thus correlates well with the salt dependent equilibrium between polymerase monomers and dimers in free solution (7). The direct involvement of polymerase dimers in the binding reaction at low salt is indicated by the characteristics of complex formation at the lacUV5 promoter. This requires only a single monomer (ref. 2 and Figure 3), or half the functional unit of polymerase activity, implying that the functional interaction between two polymerase monomers must be broken prior to complex formation. The simplest interpretation of this result is that at low salt the functional unit is a dimer which dissociates into monomers before binding to the lacUV5 promoter.

Is the observed difference between the lacUV5 and SSU1 promoters a consequence of promoter structure? The DNA fragments containing promoters differ by only 1% in length. Target size thus should not be a significant variable. A more probable source of artifact is the proximity of the tyrT promoter to the upstream end of the SSU1 and SSU2 restriction fragments. However two lines of evidence show that a tyrT promoter with 252 bp of wild type sequence 5' of the transcription startpoint can also bind two molecules of RNA polymerase (Travers, Lamond, Mace and M.L. Berman, submitted for publication). First, polymerase binds to this promoter with a sigmoidal dependence on enzyme concentration. Second, 40-50% of these polymerase-DNA complexes, visualised by electron microscopy, contain two tightly packed polymerase molecules bound in close proximity to the promoter.

The involvement of a polymerase dimer in initiation complex formation has previously been inferred for the formation of productive complexes on poly d(AT) by core polymerase but not by holoenzyme (24). However, the possible involvement of two holoenzyme molecules in promoter specific transcription is suggested by the observation that the two tandem promoters of the E. coli rRNA cistrons together direct the synthesis of

four rRNA transcripts in a single round of transcription (3,25). The sigmoid dependence of rRNA transcription on polymerase concentration in vitro (26) also indicates that a cooperative interaction between polymerase molecules may be required for rRNA synthesis. The observation that RNA synthesis from both the rRNA and tyrT promoters is unusually sensitive to increasing salt concentration (3-5) is also consistent with a requirement for polymerase dimerisation for efficient initiation.

The effect of ppGpp on polymerase-promoter complex formation parallels that of high salt in two ways. First, 100 μ M changes the functional unit of polymerase activity at low salt from a dimer to monomer (Table 1) suggesting that the nucleotide alters the equilibrium between monomer and dimer forms of the enzyme in favour of monomers. Second, ppGpp reduces the apparent binding constant of RNA polymerase to the lacUV5 promoter. At 100 μ M ppGpp and 0.05 M KCl this constant is 5×10^8 M^{-1} in comparison with calculated constants of 2×10^{10} M^{-1} and 3.7×10^8 M^{-1} 0.05 M and 0.15 M KCl respectively in the absence of ppGpp. Both results are consistent with the increased sensitivity of transcription to high salt concentrations in the presence of ppGpp (3,5). Shaner et al (27) have suggested that the extent of dimerisation of polymerase holoenzyme is determined by an anion dependent conformational transition of monomers in which the cooperative binding of up to five monovalent anions antagonises dimerisation. This transition also markedly lowers the binding affinity for many promoters (20). We suggest that the conformational change mediated by ppGpp (28) may be equivalent to this

Table 1.

Promoter	0.05 M KCl		0.05 M KCl + 100 μ M ppGpp		0.15 M KCl	
	n	pol. monomers bound	n	n	pol. monomers bound	
SSU1	0.9	1.9	1.9	1.9	-	
<u>lacUV5</u>	0.44	-	0.9	0.9	1.1	
SSU2	0.9	-	2.0	1.9	-	

Characteristics of polymerase binding to SSU1, lacUV5 and SSU2 promoters. n = slopes of plot of log (DNA bound) against log (E) from Figure 2b. Monomers of polymerase holoenzyme bound from Figure 3.

salt-induced transition with respect to its effect on both dimerisation and binding affinity.

Transcription from the wild type tyrT promoter is negatively controlled by ppGpp in vitro (18) whereas that from the mutant SSU2 tyrT promoter is positively controlled (12). By contrast ppGpp inhibits the binding of RNA polymerase to both promoters at low enzyme concentrations. To reconcile these observations we propose that ppGpp regulates more than one step in the initiation of tyrT transcription. In particular we suggest that RNA polymerase binds initially to the promoter as a dimer. This step is inhibited by ppGpp at both the wild type and SSU2 promoters and probably corresponds to inhibition of polymerase binding to the rrn promoters observed by Hamming et al (19). We show elsewhere (Travers, Lamond, Mace and M.L. Berman, submitted for publication) that this control is dependent on a sequence located upstream of the -35 region. The second step regulated by ppGpp is the recognition of the discriminator sequences immediately distal to the -10 region (12,29). This recognition may be associated with either open complex formation or initiation since no polymerase DNA contacts in this region are required for initial binding (30).

We note that a requirement for a polymerase dimer for initiation complex formation at the tyrT and other stable RNA promoters could explain the characteristic growth rate dependence of stable RNA synthesis in bacteria. The activity of such promoters would be dependent on the square of polymerase holoenzyme concentration, whereas the activity of promoters requiring only a single monomer would be directly proportional to polymerase concentration. The concentration of RNA polymerase in vivo increases in proportion with growth rate (31). Therefore a dimer requirement for initiation at stable RNA promoters and a monomer requirement for most mRNA promoters would result in the rate of production of stable RNA being proportional to the square of the growth rate and that of bulk messenger RNA being directly proportional to growth rate. This corresponds to the pattern observed in vivo (32,33). Moreover this characteristic pattern is determined by DNA sequences associated with promoters (34). In addition regulation of dimerisation by ppGpp during the stringent response could potentially result in a rapid switch to a population of polymerase molecules consisting principally of monomers. Such a switch could again account for the alteration of the pattern of transcription of the bacterial genome upon aminoacid starvation.

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