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Interaction between RNA polymerase and a ribosomal RNA promoter of E.coli

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ABSTRACT

The interaction between RNA polymerase and the E. coli ribosomal (r) RNA promoter(s) of the rrnE operon has been studied by the filter-binding method. The extent of complex formation between RNA polymerase and rrnE promoter(s) is salt-dependent; ppGpp specifically inhibits interaction of RNA polymerase with the rrnE promoter(s). A tentative model is proposed for the molecular events in the early steps of rRNA initiation: a transition of the primarily formed, labile RNA polymerase-rRNA promoter complex to a more stable form is the determining step. This step is salt-sensitive; ppGpp acts on this "isomerization".

INTRODUCTION

In E. coli there are probably seven ribosomal (r)RNA operons per chromosome (1,2), comprising about 1% of the genome. Under various nutritional conditions the number of ribosomes per genome is approximately proportional to the growth rate (3). At maximal growth rate, when about 70% of all RNA made is rRNA, RNA polymerase molecules are packed close to the physical limit on the ribosomal RNA operons: in other words, the rate of rRNA synthesis is then governed by the rate of elongation which apparently can be matched by the initiation frequency. Also in vitro, in a purified system containing only DNA and RNA polymerase as macromolecular components, we found maximal packing of RNA polymerase molecules on the ribosomal RNA operons under optimal conditions (4); of course, the rate of rRNA synthesis is lower in vitro than in vivo because of the lower elongation rate. So, under optimal conditions initiation of rRNA transcription is very efficient in vivo as well as in vitro.

Specific inhibition of rRNA synthesis occurs in bacteria

under conditions of amino acid starvation. It has been shown that the nucleoside tetraphosphate guanosine-3'-diphosphate, 5'-diphosphate (ppGpp) is involved in this so-called stringent response (5). This nucleotide specifically inhibits rRNA synthesis in vitro, in a purified system containing only E. coli DNA and RNA polymerase as macromolecular components (6). The K_i found in vitro was similar to that calculated for the stringent response in vivo. We now know that ppGpp specifically inhibits transcription of at least six rRNA operons (4,7 t/m 10), and that ppGpp acts on the initiation step (4,8).

The very efficient initiation of rRNA synthesis and the specific inhibitory effect of ppGpp, point to special features of the rRNA promoters, the DNA regions where RNA polymerase recognizes signals for initiation of rRNA transcription. Binding of RNA polymerase to a promoter is by definition the first step in initiation. To study this binding, we measured complex formation between RNA polymerase and a restriction fragment of λmetA20 DNA. This transducing-phage DNA contains the rrnE operon (15); we chose a restriction fragment that contains no other than the rrnE promoter. Recently it was shown that transcription of the rrnE operon starts from two promoters in tandem (10). Tandem promoters have also been shown for rrnA (10), rrnB (12), rrnD and rrnX (13) operons. In this paper we present experiments on the interaction between RNA polymerase and the rrnE promoter(s): the effect of salt concentration and of ppGpp was studied. We propose a tentative model for the first steps in initiation of rRNA synthesis.

MATERIALS AND METHODS

Strain NO 1821 [AB 2569(λcI857S7,λmetA20)] was a gift of Dr. M. Nomura; strain W3350/pKB252 was a gift of Dr. H. Schaller. Restriction endonucleases: EcoRI was purchased from Boehringer (Mannheim), HhaI, MboII (from New England Biolabs) and HpaII, HaeIII were kindly provided by Mr. P. van Wezenbeek. Polynucleotide kinase from phage T_4 and alkaline phosphatase from calf intestine were from Boehringer (Mannheim). RNA polymerase was isolated according to Burgess and Jendrisak (22). Agarose (electrophoresis purity reagent), and hydroxyapatite

(Bio-Gel HTP) were from Bio-Rad, acrylamide and sarkosyl were from SERVA. [γ-^{32}P]ATP(3000Ci/mmol) was from The Radiochemical Centre Amersham, nitrocellulose membrane filters (ø 15µ) were from Sartorius (Göttingen, GFR).

Preparation of λmetA20 DNA and of plasmid pKB252.

λmetA20(15) was grown by thermal induction of the lysogen and separated from helper phage according to Miller (16). Phage DNA was stored in 10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, at 4°C. Plasmid pKB252, containing P^rlac L8UV5(17) was isolated as described in Ref. 18. Plasmid DNA was stored in 10 mM Tris acetate, pH 7.8; 0.1 mM EDTA, at -20°C.

Purification of restriction fragments.

Digestion of DNA with restriction enzymes was done at 37°C in a buffer containing 10 mM Tris-HCl pH 7.6; 10 mM $MgCl_2$; 10 mM βmercaptoethanol; 50 mM NaCl for HhaI, HpaII, HaeIII, EcoRI and with 6 mM KCl instead of 50 mM NaCl for MboII.

For purification of restriction fragments the method of Tabak and Flavell (19) was used. EcoRI digested λmetA20 DNA (1.5-2 mg) was loaded on a 0.7% agarose gel; before loading on the gel EDTA pH 7.5 was added till a final concentration of 20 mM and half a volume agarose beads (0.2% agarose in 10 mM Tris-HCl, pH 7.5; 20 mM EDTA; 10% glycerol; bromophenolblue) was added (20). Dimensions of the gel were 17 x 18 x 2.5 cm, slot 12 x 0.15 x 2.3 cm. Electrophoresis buffer contained 40 mM Tris acetate; 20 mM sodium acetate (pH 7.7); 1 mM EDTA; 0.5 µg/ml ethidium bromide. Electrophoresis first dimension: overnight 50V/gel. DNA bands were visualized by illumination with an UV lamp. The fragment to be purified was electrophoresed into hydroxyapatite, DNA was eluted from hydroxyapatite as described in Ref. 19, ethidium bromide was removed by isoamylalcohol extraction, and after ethanol precipitation DNA was dissolved in 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA and stored at 4°C.

Labelling of restriction fragments.

Purified restriction fragment or a total digest was labeled with ^{32}P at the 5'end according to Maxam and Gilbert (21) with some minor modifications.

Binding experiments

Standard assay conditions: RNA polymerase and a ^{32}P-end-

labeled purified restriction fragment, or a total digest were incubated in binding buffer (20 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 0.1 mM dithiothreitol; 0.1 mM EDTA; 5% glycerol, see Ref. 14) with 200 µg BSA per ml and 40 mM or 120 mM KCl in a total volume of 30 µl at 37°C. After 5 min incubation, two volumes of warm binding buffer, containing sonified, denatured calf thymus DNA (final concentration 30 µg/ml) were added. The final KCl concentration always became 40 mM. Incubation was continued for 5 min and the mixture was filtered through nitrocellulose membrane filters. The filters were dried, put into a scintillation vial, scintillation liquid (4 g of diphenyl-oxazole and 80 mg of p-bis-(o-methylstryryl)-benzene/liter of toluene) was added and radioactivity was counted in a Nuclear Chicago scintillation counter, Mark II. An assay blank without RNA polymerase was subtracted (generally 8% of DNA input). When a mixture of restriction fragments was tested, small nitro-cellulose filters (about 3 x 3 mm) were used. Filters were eluted three times with 10 µl of 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 0.1% sarkosyl. About 90% of the radioactivity was eluted from the filter. Half a volume agarose beads (see above) was added and restriction fragments were separated by polyacrylamide slab-gel electrophoresis (5% acrylamide), electrophoresis buffer 90 mM Tris borate pH 8.3; 2.5 mM EDTA.

Other methods.

RNA synthesis, hybridisation, preparation of ppGpp were as described in a previous paper (4). All buffers were prepared in double distilled water.

RESULTS AND DISCUSSION

To understand effects of control elements on rRNA synthesis it is essential to identify the molecular events in initiation of rRNA transcription. The first step in initiation of rRNA transcription is binding of RNA polymerase to a ribosomal RNA promoter. We studied the interaction between RNA polymerase and the promoter of the rrnE operon by measuring complex formation between RNA polymerase and the 1.4 kb EcoRI fragment of λmetA20 DNA. The 1.4 kb EcoRI fragment contains the promoter region of the rrnE operon and 670 basepairs (bp) of m16S sequence (Fig. 1,

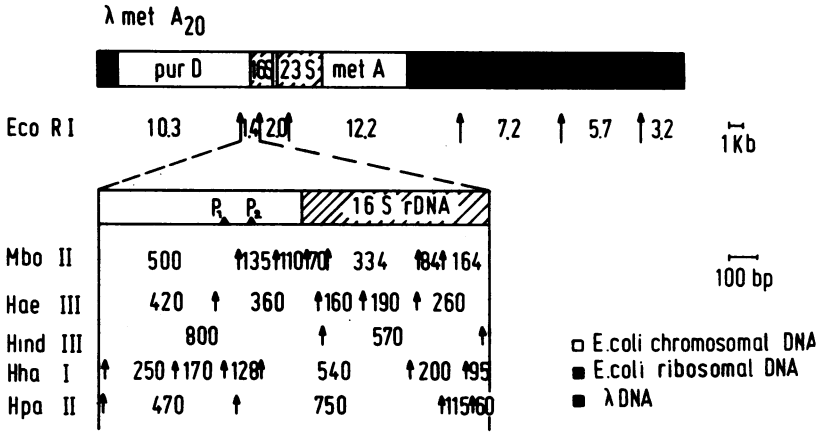

Figure 1. Restriction map of λmetA20 DNA and detailed map of the 1.4 kb EcoRI fragment.
The data were taken from Ref. 11.

Refs. 28,29). Recently the sequence of the rrnE promoter region was determined (11), and transcription of the rrnE operon was shown to start from two promoters in tandem (10). The starts designated P_1 and P_2 are bp 283 and 174, respectively, upstream from the 5' end of m16S (10,11). The detailed map of the 1.4 kb EcoRI fragment shows that smaller fragments which contain only P_1 or P_2 intact can be prepared. These fragments allow assays of complex formation with either promoter.

For measuring complex formation we used the filter-binding technique according to Seeburg et al (14): RNA polymerase is incubated with the labeled 1.4 kb EcoRI fragment or a restriction enzyme digest of this fragment. The binding reaction is stopped by the addition of single-stranded DNA which traps all free RNA polymerase molecules; because of rapid dissociation of non-specific or loose RNA polymerase-DNA complexes,also non-specifically bound RNA polymerase molecules are trapped by single-stranded DNA. Only DNA fragments which have bound at least one RNA polymerase molecule in a stable complex will be retained on a nitrocellulose filter which retains RNA polymerase but not free double stranded DNA.

Formation of stable RNA polymerase-rrnE promoter complexes

Fig. 2 shows "titration" curves of the 1.4 kb EcoRI fragment with increasing RNA polymerase concentrations at two different salt concentrations. It is apparent that at 120 mM a "steady state" is reached and that the extent of binding over the whole range of RNA polymerase concentrations is much lower than at 40 mM KCl.

Figure 3 shows experiments in which the rate of stable complex formation was determined at the salt concentrations mentioned above. Fig. 3A shows the time course for the first five minutes; in another experiment (Fig. 3B) we incubated RNA polymerase and the 1.4 kb fragment for longer times before single-stranded DNA was added. From these data, which were confirmed in a number of similar experiments, we may conclude that at 120 mM KCl formation of a stable complex between RNA polymerase and the 1.4 kb fragment reaches a plateau value. At 40 mM KCl the extent of binding still increases between 5 and 60 minutes but in similar incubations for longer periods the binding never exceeded 60% of DNA input. This end value was not due to partial leakage

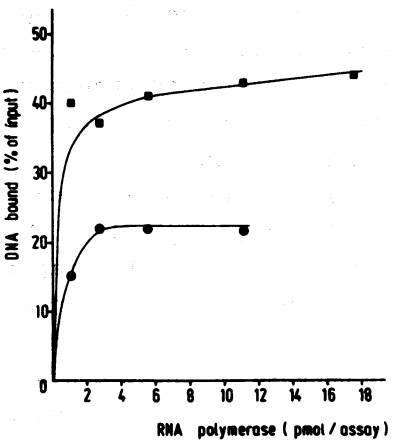

Figure 2. Binding of RNA polymerase at different conditions. RNA polymerase and ^{32}P-end-labeled 1.4 kb EcoRI fragment (0.08 pmol/assay, spec. act. 30 Ci/mmol) were incubated under standard assay conditions at 40 mM (—■—) or 120 mM KCl (—●—). Treatment after complex formation was as described in Materials and Methods.

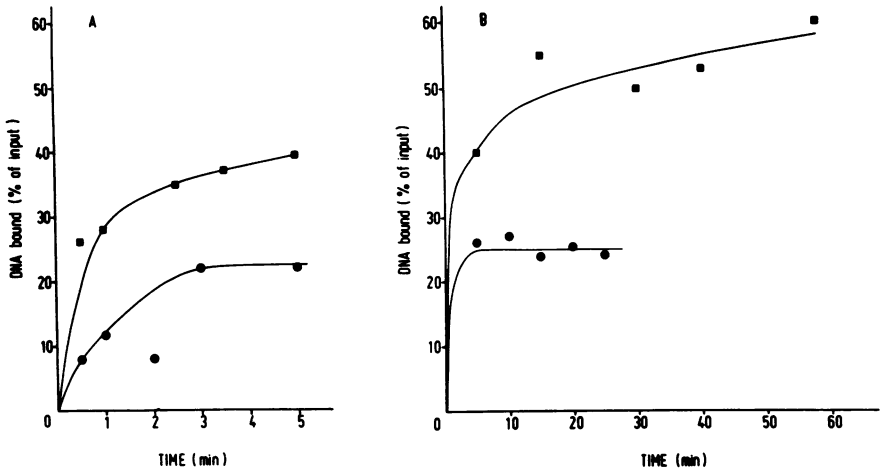

Figure 3. Time dependence of complex formation.
RNA polymerase (in A 2.8 pmol/assay, in B 5.6 pmol/assay) and the ^{32}P-end-labeled 1.4 kb EcoRI fragment (0.06 pmol/assay; spec. act. 40 Ci/mmol) were incubated together under standard assay conditions, at 40 mM (—■—) or 120 mM KCl (—●—), for the periods indicated. Further treatment was as described in Materials and Methods.

of RNA polymerase through the filter (enzymatically tested) nor to heterogeneity of the DNA fragment (data not shown). The levels of binding reached at 40 mM and 120 mM KCl were independent of the RNA polymerase preparations used which had been isolated according to different procedures (4,22,23).

The lower extent of binding at higher salt concentration fits with the rRNA transcription characteristics: in vitro at 120 mM KCl, rRNA initiation frequency is lower than at 40 mM KCl (4).

The non-attainment of 100% binding at 40 mM KCl upon incubation of the DNA fragment with a large excess of RNA polymerase for five minutes (Fig. 2) and also for longer periods of time (Fig. 3B) exclude a simple one-step equilibrium for the binding reaction. Our results must be due to a more complex mechanism. The relatively most simple one is a mechanism with two complexes, I and II, according to the following scheme:

DNA + RNA polymerase $\rightleftharpoons$ complex I $\rightleftharpoons$ complex II

A similar scheme for the early steps in initiation has been

postulated earlier by several workers (for a review see Ref.24); mostly complex I was considered as the "closed", and II as the "open" complex.

We assume that complex I forms and decays quickly. It will therefore rapidly decay during incubation with single-stranded DNA and thus not be measured with the filter-binding technique used. In the model the difference in plateau values found at different salt conditions must be explained in the following manner: the higher KCl concentration affects the equilibrium between I and II by decreasing the rate of formation or increasing the rate of decay of complex II, or both. Previously we have postulated the existence of a two-step binding to explain the effect of salt and ppGpp on the initiation of rRNA transcription (4,8). Also the observation that high glycerol concentrations enhance the extent of binding at 120 mM KCl, argues in favour of the proposed model; glycerol is known to lower the melting temperature of DNA, and therefore it will oppose the effect of higher salt concentrations on the opening of the complex.

Dissociation of complexes

In the experiments shown in Figure 4, complexes between RNA polymerase and 1.4 kb fragment were formed at 40 mM and at 120 mM KCl; the stability of the complexes at these salt concentrations was determined by incubating them with single-stranded DNA at 40 mM, and at 120 mM KCl,respectively. When we incubate RNA polymerase with the 1.4 kb fragment presumably three different types of complex are formed (24): non-specific complexes (non-promoter complexes, due to general affinity of RNA polymerase for DNA), and the promoter complexes complex I and complex II. Because the non-specific complexes and complex I dissociate rapidly, their concentration will soon drop to zero upon incubation with single-stranded DNA. Thus only fragments which have bound a RNA polymerase molecule at the promoter site in complex II form will be retained on the filter.

Fig. 4 shows that complex II dissociates very slowly at 40 mM KCl independent of the salt concentration at which it was formed. This slow dissociation allows the measurement of the formation of complex II. Thus under standard assay conditions

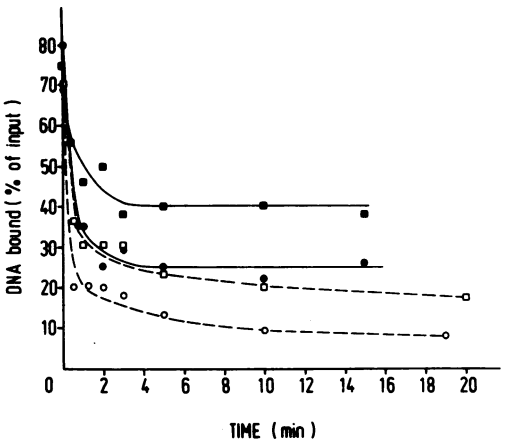

Figure 4. Dissociation of complexes.
RNA polymerase (5.6 pmol/assay) and the ^{32}P-end-labeled 1.4 kb EcoRI fragment (0.056 pmol/assay; spec. act. 50 Ci/mmol) were incubated under standard assay conditions at 40 mM (squares) or 120 mM KCl (circles). After complex formation, incubation with calf thymus DNA was performed for the periods indicated, in the presence of 40 mM (closed symbols) or 120 mM KCl (open symbols).

40% of input DNA has entered complex II at 40 mM KCl whereas only 25% has done so at 120 mM KCl.

In 120 mM KCl complex II is unstable whether it was formed at 40 mM or at 120 mM KCl. Apparently salt enhances the rate of decay of complex II. When ln (DNA bound) is plotted versus time, straight lines are obtained; we calculated a first-order constant of about 0.12 min^{-1} for complex II formed at 40 mM as well as at 120 mM KCl. This value corresponds with a $t_{\frac{1}{2}}$ of about 6 min. Apparently the complexes behave similar in this respect. Thus high salt concentration decreases the amount of complex II by accelerating the decay, independent of the salt concentration at which the complex was formed.

Binding to restriction fragments of the 1.4 kb EcoRI fragment.

Since the results presented above were obtained with the complete 1.4 kb EcoRI fragment the contribution of the individual promoters cannot be assessed. Therefore, we digested the fragment with suitable restriction enzymes in order to

generate fragments containing only promoter site P_1 or P_2. In view of the known physical map (Fig. 1) and the base sequence of the important region (11) we chose the enzymes MboII, HpaII, HaeIII and HhaI. Fig. 5 shows the exact size and location of the fragments. We performed binding experiments with the different digests under standard assay conditions at 40 mM KCl, and determined which sub-fragments had bound RNA polymerase in a stable form.

We found stable binding of RNA polymerase to the following fragments from the tandem promoter region (Figs. 1,5): MboII/500, HpaII/470, HpaII/750, HaeIII/360 and HaeIII/420. Since no other promoters are located upstream from promoter P_1 (10), MboII/500 and HpaII/470 should contain intact promoter P_1,while HaeIII/360 and HpaII/750 should comprise promoter P_2 intact and active (P_1 is not functional on HaeIII/360, see Ref. 10). HaeIII/420 which does not comprise the start site of P_1 transcription probably contains a sufficient part of the binding site of promoter P_1.

Unexpectedly we also found binding to fragments MboII/334 and HhaI/540 (Figs. 1,5). Apparently a binding site in the 16S region exists; thus binding to HpaII/750 is due either to P_2 intactness or to the binding site in the 16S region or both. The presumed 16S binding site should overlap the HaeIII 160/190

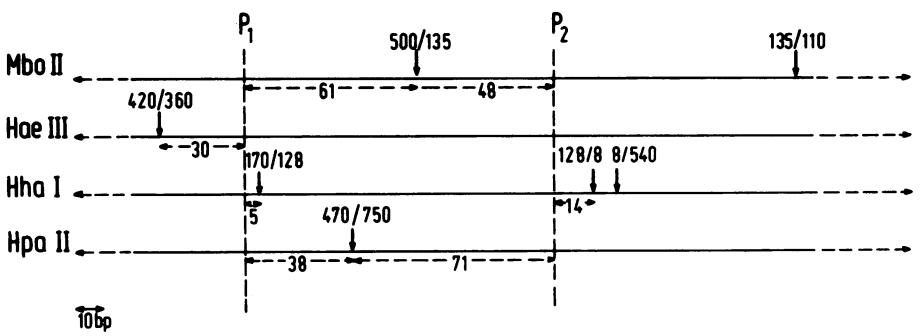

Figure 5. Restriction map of the promoter region of rrnE.
P_1 and P_2 indicate the starts of transcription from the two tandem promoters; P_1 is basepair (bp) 283, P_2 is bp 174 upstream from the 5' end of m16S (Ref. 10). For some restriction enzymes the distance from a nick to P_1 or P_2 is indicated in bp.

cleavage site since neither of these fragments is able to bind RNA polymerase in a stable complex. In further experiments binding of RNA polymerase to P_1 and P_2 was assessed by measuring binding of RNA polymerase to MboII/500 and HaeIII/360, respectively.

Remarkably, no binding was found to MboII/135 and to HhaI/128 after 5 min incubation with excess RNA polymerase. The minimum length of DNA upstream and downstream from the start site of transcription, which is required for promoter function is unknown. Gilbert reported that more than 35 bp upstream are necessary (25) with a number of promoters, Brown et al. reported for the trp promoter more than 39 bp (26); moreover, about 40 bp centered round the start site of transcription are protected against DNase by RNA polymerase (for review see Ref. 25). With MboII/135 the piece of DNA upstream from the start site of P_2 transcription (48 bp) is probably too small to allow stable complex formation; perhaps the A-T rich region 50-60 bp upstream from the start site of P_2 transcription is necessary for stable binding. With HhaI/128, the piece of DNA downstream from the start site of P_2 transcription (14 bp) might be too small for stable complex formation.

The effect of ppGpp.

To investigate the effect of ppGpp on binding of RNA polymerase to P_1 and P_2 in tandem, and to P_1 and P_2 separately, we performed binding experiments with a mixture of 1.4 kb EcoRI fragment, a HaeIII digest and a MboII digest of this fragment in the presence and absence of ppGpp. Figure 6 shows the effect of ppGpp on the binding of RNA polymerase to 1.4 kb EcoRI, MboII/500 and HaeIII/360 and shows a clear inhibitory effect of ppGpp on the extent of complex formation between RNA polymerase and the fragments which contain ribosomal RNA promoter(s). In the same experiment we found no effect of ppGpp on the binding to MboII/334 (16S). Thus ppGpp specifically acts on promoters P_1 and/or P_2 when in tandem, and on either of them when separate.

Tentative data from experiments with the 1.4 kb EcoRI fragment only, suggest that ppGpp decreases the stability of complex II in our model, i.e. ppGpp increases the decay rate.

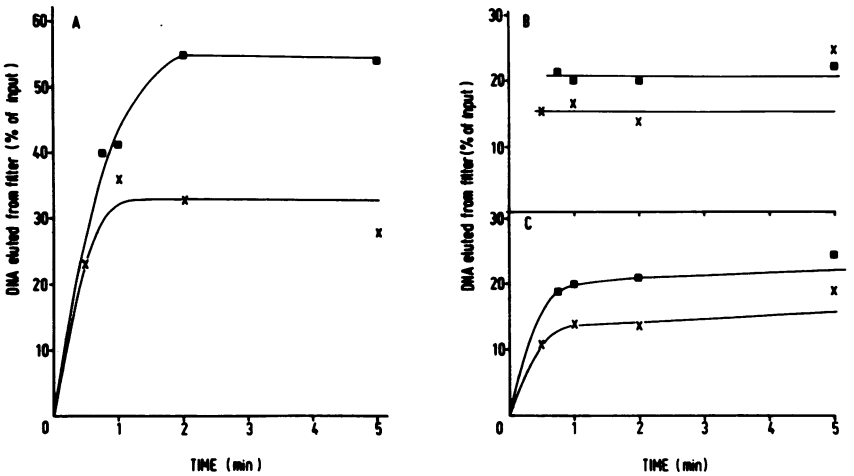

Figure 6. Effect of ppGpp on complex formation.

RNA polymerase (26.4 pmol/assay) was incubated with a mixture of ^{32}P-end-labeled 1.4 kb EcoRI fragment (0.05 pmol/assay, spec.act. 48 Ci/mmol), a HaeIII digest (0.13 pmol digested 1.4 kb fragment/assay; total activity/assay 0.011 µCi) and a MboII digest of this fragment (0.16 pmol digested 1.4 kb fragment/assay; total activity 0.014 µCi/assay), under standard assay conditions (40 mM KCl) in the presence (-✕-) or absence (-■-) of ppGpp (0.8 mM). Incubation was for the periods indicated and further treatment was as described in Materials and Methods.

A. Complex formation between RNA polymerase and 1.4 kb EcoRI fragment (P_1 and P_2 in tandem).
B. Complex formation between RNA polymerase and MboII/500 (promoter P_1)
C. Complex formation between RNA polymerase and HaeIII/360 (promoter P_2)

Whatever the mechanism, we could demonstrate a direct effect of ppGpp on the interaction of RNA polymerase to DNA which comprises rRNA promoter(s) of the rrnE operon of E. coli. The inhibitory effect of ppGpp on RNA polymerase binding to the ribosomal RNA promoter(s) of the rrnE operon is in agreement with the effect of ppGpp on transcription from the rrnE promoter(s) (10).

As a control we measured the effect of ppGpp on the binding of RNA polymerase to a 203 bp EcoRI fragment from pKB252 (Ref. 17), containing P^rlac L8UV5, and found a twofold stimulation of complex formation. Again this is in agreement

with the ppGpp effect on transcription of the lac operon (27) from P^rlac L8UV5.

GENERAL DISCUSSION

Our results clearly show that the ribosomal promoter(s) of the rrnE operon can strongly bind to RNA polymerase. The results from binding experiments with the 1.4 kb EcoRI fragment are complicated by the presence of two promoters in tandem -an apparently general feature of rRNA operons (10,12,13)- and by the surprising presence of a "promoter-like" binding site in the m16S region. The latter binding site will not have contributed essentially to the characteristics of RNA polymerase binding to the 1.4 kb fragment because the binding of RNA polymerase to the HindIII/800 fragment shows the same characteristics (data not shown); the HindIII/800 DNA contains both promoters, but no 16S region. The clear ppGpp effect on the binding of RNA polymerase to the 1.4 kb fragment and the sub-fragments containing the promoter sites, while no effect of ppGpp was found on the binding to the 16S binding site, leads to the same conclusion.

The effects of salt and ppGpp are in agreement with the transcription results (4,10) again showing that we were dealing with the ribosomal promoter(s). Whether ppGpp has still other effects on initiation than on the RNA polymerase-promoter binding observed here, has to be studied; anyhow, its effect on the binding proves its direct molecular participation.

We propose a simple model for early events in initiation of rRNA transcription which can explain our present results. It may turn out to be an oversimplification when more data are obtained. We have shown that rrnE promoter(s) can strongly bind to RNA polymerase, but comparisons with other E. coli promoters will have to be made to determine whether ribosomal RNA promoters are as extremely strong in binding as they are in transcription. The possible function of tandem promoters is at this stage unclear, nor do we know whether both promoters on the same DNA molecule can bind simultaneously. Further experiments may throw some light on the tandem promoter role.

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