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E. coli RNA polymerase - rRNA promoter interaction and the effect of ppGpp

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ABSTRACT

The interaction between RNA polymerase and the *E. coli* r(ibosomal) RNA promoters of the rrnX and rrnE operon was studied with the filter-binding technique. Quantitative differences were observed between the rrnX and rrnE promoters: stable rrnX promoter complexes are formed faster, and are less sensitive towards heparin and salt than stable rrnE promoter complexes. The effect of ppGpp, the specific inhibitor of rRNA synthesis, on rrn promoter complex formation was studied. In the presence of ppGpp complexes are formed which cannot be trapped in a transcription complex by addition of the start nucleotides, and are therefore considered to be non-productive. A tentative model for the action of ppGpp is proposed.

INTRODUCTION

When *E. coli* is subjected to starvation of a required amino acid immediate and abrupt cessation of the synthesis of rRNA results. It has been shown that the nucleoside tetraphosphate guanosine-3'-diphosphate, 5'-diphosphate (ppGpp) is involved in this so-called stringent response (1).

Experiments *in vitro* have implicated ppGpp as a direct negative effector of rRNA synthesis (2,3,4). We know that ppGpp acts on the initiation step (3,5) and that ppGpp decreases complex formation between RNA polymerase and a rRNA promoter (6). The molecular mechanism of its action is still unknown.

In *E. coli* there are probably seven ribosomal RNA operons per chromosome (7,8,9). The DNA sequences of the promoter regions of five rRNA operons have been determined: rrnA, rrnE (10), rrnD, rrnX (11) and rrnB (12). The promoter regions of these operons turned out to be non-identical. A recent article about rRNA promoter sequences contains a detailed comparison of all sequences

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known (12). In vitro transcription experiments have demonstrated (a) that each operon has two promoters in tandem with their initiation sites at about 280 and 170 base pairs upstream from the 5' end of the m(ature) 16S rDNA, and (b) that the transcript from the first promoter starts with ATP (in rrnA, B, E and X) or GTP (in rrnD) whereas the transcript from the second promoter starts with CTP in all five of these operons (11,13,14).

Although the rRNA promoter regions are different, ppGpp specifically inhibits transcription of each of the operons (3,5,13, 15). Travers has recently identified a highly conserved region in a number of promoters, which may turn out to be important for the stringent control (16).

In a previous paper (5) quantitative differences in salt optimum and ppGpp sensitivity of the RNA synthesis in vitro were reported between the ribosomal RNA operons rrnB, rrnD and rrnX. These differences are at an initiation step (5) and must be due to differences in the promoter sequences (12). When these experiments were pursued with a fourth operon, rrnE, lying on phage λmetA20 (17) DNA, we found its transcription to be more sensitive towards high salt than RNA synthesis on the rrnX operon on phage λd5ilv (15) DNA (unpublished experiments). Since both promoter regions are very similar and only differ beyond 20 basepairs upstream from the first transcription initiation site P_1 (10,12) they offer the opportunity to identify the relevant sequences. We therefore decided to compare the interaction of RNA polymerase with either of both operons, rrnE and rrnX.

In this paper we present experiments on complex formation between RNA polymerase and these promoters and the effect of salt, heparin and ppGpp. Our experiments led to a tentative model for the action of ppGpp.

MATERIALS AND METHODS

Strain NO 1821[AB 2569(λcI857S7,λmetA20)] was a gift of Dr. M. Nomura; E. coli NF 955 (thr^-, leu^-, thi^-, ileC, λcI857S7, d5ilv rrn, λcIb2) was a gift of Dr. P. Jørgensen (15). Restriction endonucleases: EcoRI, BsuRI, AluI and HindIII were purified according to Greene et al.(18); HindIII was subjected to an additional Cibacron blue F3GA agarose column chromatography

according to Ref. 19. The enzyme was free from HindII activity. HpaII, Sma, HhaI, MboII were from New England Biolabs. RNA polymerase was isolated according to Burgess and Jendrisak (20). Nucleoside triphosphates and GDP were purchased from Boehringer. ppGpp was obtained from ICN Pharmaceuticals and was further purified by DEAE-Sephadex A25 chromatography as described in Ref. 21. Heparin was from Sigma.

Preparation of λmetA20 DNA and of λd5ilv DNA.

λmetA20 (17) and λd5ilv (15) were grown by thermal induction of the lysogen. Separation from helper phage and DNA extraction was as described by Miller (22). Phage DNA was stored in 10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, at $4^{o}C$.

Purification of restriction fragments.

Digestion of DNA with restriction enzymes was carried out at $37^{o}C$ in a buffer containing 10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 1 mM DTT; 50 mM NaCl for HindIII, EcoRI, AluI, HhaI; in the same buffer containing 150 mM NaCl for BsuRI; without NaCl for HpaII, MboII; with 14 mM KCl instead of NaCl for Sma. Digestion with AluI was always incomplete.

Purification of the 1.4 kb EcoRI fragment of λmetA20 DNA, the 2.5 kb HindIII fragment of λd5ilv DNA, containing the ribosomal RNA promoters of the rrnE operon and rrnX operon, respectively, was as described earlier (6). For purification of sub-fragments of the 1.4 kb and 2.5 kb fragments, restriction enzyme digests of these fragments were prepared, treated with phenol, ethanol precipitated, washed, dried under vacuum and dissolved in 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA. The total digest was labeled with ^{32}P at the 5' end according to Maxam and Gilbert (23) with some minor modifications, and layered on a polyacrylamide slab-gel (5% polyacrylamide). Electrophoresis was performed at constant voltage (120V) for 3 hrs in 90 mM Tris borate pH 8.3; 2.5 mM EDTA. Fragments were visualized by autoradiography and gel regions containing the fragments were cut out, put in a dialysis bag together with about 5 ml of buffer, containing 40 mM Tris acetate; 20 mM sodium acetate (pH 7.7); 1 mM EDTA; 0.2% SDS and DNA was recovered by electro-elution (30V, 240 mA for 50 hrs in the same buffer) and further purified and concentrated by using a small DE 52 column which was equilibrated with 50 mM Tris-HCl, pH 7.5;

5 mM EDTA; 100 mM NaCl. DNA was eluted from the column with the same buffer containing 1.5 M NaCl. If necessary the purified fragment was again terminally labeled to obtain a higher specific activity.

Binding experiments.

RNA polymerase and a ^{32}P-end-labeled purified restriction fragment or a mixture of restriction fragments were incubated under standard assay conditions (6) at low ionic strength (40 mM KCl). During incubation with single stranded calf thymus DNA, the stable promoter complexes are retained. When a mixture of restriction fragments was tested, fragments bound were eluted from the filter and separated by electrophoresis. The details of the procedure are described in Ref. 6.

Heparin experiments.

For complex formation RNA polymerase and the ^{32}P-end-labeled 2.5 kb HindIII fragment (rrnX) or the 1.4 kb EcoRI fragment (rrnE) were incubated under standard assay conditions mentioned above. After 5 minutes incubation, one volume of warm binding buffer (with 40 mM KCl), containing sonified, denatured calf thymus DNA (final concentration 46 μg/ml) was added. Incubation was continued for 5 minutes and then half a volume of warm binding buffer (with 40 mM KCl), containing heparin (final concentration 55 μg/ml, unless otherwise indicated) was added. Incubation was continued for different periods of time. Further treatments were as described in Materials and Methods of Ref. 6.

Other methods.

All other materials and reaction conditions were as described in Ref. 6.

RESULTS AND DISCUSSION

Topography of promoter fragments

To study the interaction of RNA polymerase with the rrnE and rrnX promoters we isolated a 1.4 kb EcoRI fragment from λmetA20 DNA, containing the rrnE promoter region (10) and a 2.5 kb HindIII fragment from phage λd5ilv DNA containing the rrnX promoter region (25). The topography of both fragments with the start sites of the rRNA promoters, P_1 and P_2 aligned is depicted in Fig. 1. The 1.4 kb rrnE fragment contains 670 bp of the m16S re-

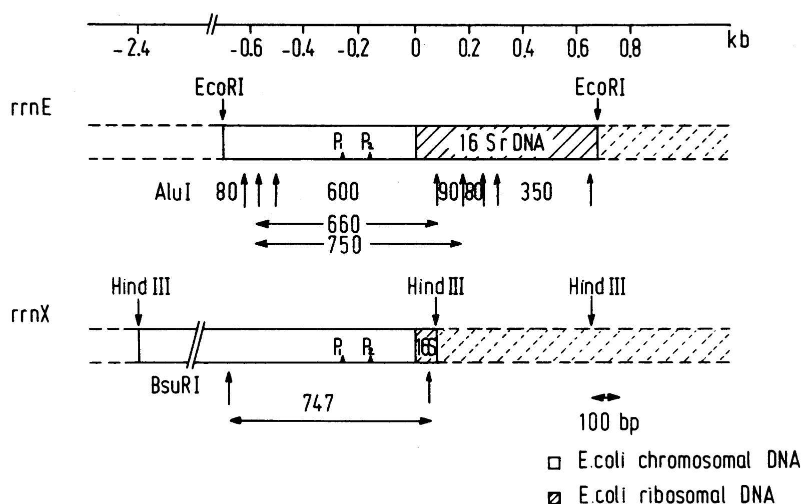


Figure 1. Detailed maps of the 1.4 kb EcoRI fragment (rrnE) and the 2.5 kb HindIII fragment (rrnX).
The data are mainly obtained from Refs. 10,11.

gion and extends 450 bp upstream from P_1 as has been described in detail in a previous paper (6). The localisation of the 2.5 kb rrnX fragment especially the number of m16S basepairs contained in the fragment, was examined further. For a number of ribosomal RNA operons, rrnA, rrnE (6,10) and rrnB (9,14,26), HindIII sites have been found at position 80 and position 647 of the m16S region. The rrnX and rrnD have been reported to contain only the HindIII site at position 647 (25,27). This might be due to limited heterogeneity among the various rRNA operons (28). Another possibility is that the HindIII cleavage site at position 80 of the m16S region of the rrnX operon was overlooked.

To find the exact boundaries of the 2.5 kb rrnX fragment we performed detailed restriction mapping and conclude that it contains only 80 bp of the m16S region too. We arrived at this conclusion by labelling the 2.5 kb rrnX fragment terminally and measuring the length of the labeled fragments after further digestion with a number of restriction enzymes. As can be seen in Table I the size of the fragments obtained is in agreement with the presence of a HindIII site at position 80 in the m16S region.

Table I. Restriction enzyme analysis of the 2.5 kb rrnX fragment.

	Size of the fragments (basepairs)		
	expected if HindIII site at position 80	expected if HindIII site at position 647	obtained
Restriction enzyme:			
AluI	240	360	220 + 1050
Sma	2500	26	2500
HpaII	326	26	340 + 1200
HhaI	370	75	370 + 520
MboII	62	140	60 + 700

Digestion of ^{32}P-end-labeled 2.5 kb rrnX fragment (0.05 pmol/assay, spec. act. 182 Ci/mmol) with the restriction enzymes indicated and separation of the fragments were as described in Materials and Methods. The size of the labeled fragments obtained, is given, as is the size of the labeled fragments expected if the 2.5 kb fragment extends still until bp 647 in the m16S region (26) or until bp 80 in the m16S region (11,25,26).

The 2.5 kb rrnX fragment extends some 2 kb upstream from the initiation site P_1.

Interaction between RNA polymerase and the promoters of the rrnX and rrnE operon.

In preliminary experiments with the 2.5 kb HindIII fragment of λd5ilv DNA (rrnX) we determined some characteristics of its complex formation, by using the filter-binding technique (6,24). The extent of complex formation is salt-dependent and is (at 120 mM KCl) enhanced by addition of glycerol. The guanosine tetraphosphate, ppGpp, specifically inhibits the interaction between RNA polymerase and the rrnX promoter. The characteristics of the rrnX promoter are qualitatively very similar to those of the rrnE promoter, which have been described in detail previously (6). However we find quantitative differences: the rrnX promoter complexes are formed somewhat faster than the rrnE promoter complexes, the first order decay constant at 120 mM KCl for rrnX promoter complexes is half or less than half the first order decay constant for rrnE promoter complexes (6).

To exclude trivial differences in the rate of stable complex formation introduced by contaminants in the DNA preparation, we studied complex formation with both promoters in one and the same incubation mixture. Fig. 2A shows that stable complex formation between RNA polymerase and the 2.5 kb rrnX fragment is indeed faster than complex formation between RNA polymerase and the 1.4 kb rrnE fragment.

The fragments used thus far are rather long, and will contain sequences that have no function in rrn promoter recognition and/or transcription. This particularly holds for the 2.5 kb HindIII fragment with the rrnX promoters. The fragment extends so far upstream (Fig. 1) that it could possibly contain additional (non-ribosomal RNA) promoters. To exclude that the binding of RNA polymerase to the 2.5 kb rrnX fragment is due to such a putative promoter, we trimmed the fragments with restriction enzymes leaving the ribosomal promoters and limited stretches of neigh-

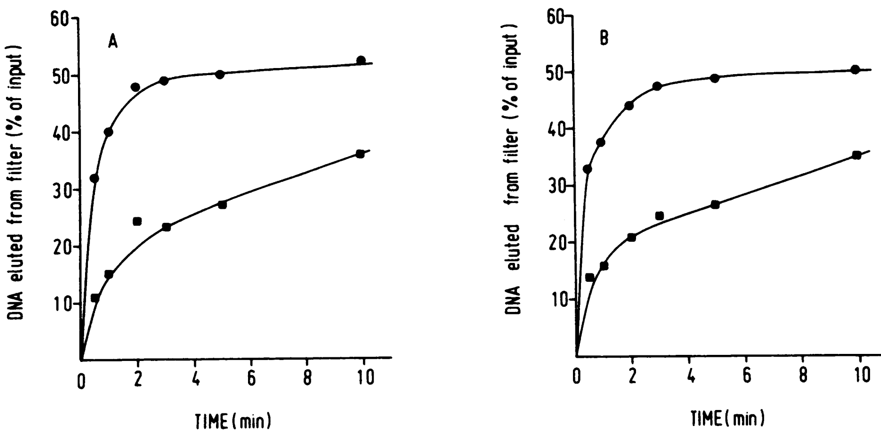


Figure 2. Time dependence of complex formation.

RNA polymerase (2.9 pmol/assay) was incubated for various periods of time under standard assay conditions (40 mM KCl) with a mixture of ^{32}P-end-labeled fragments.
Panel A: 1.4 kb rrnE fragment (0.1 pmol/assay, spec. act. 45 Ci/mmol) and 2.5 kb rrnX fragment (0.06 pmol/assay, spec. act. 68 Ci/mmol). Panel B: 660 bp rrnE fragment (0.03 pmol/assay, spec. act. 74 Ci/mmol) and 747 bp rrnX fragment (0.03 pmol/assay, spec. act. 176 Ci/mmol). Squares: Complex formation between RNA polymerase and the rrnE promoter fragment. Circles: Complex formation between RNA polymerase and the rrnX promoter fragment.

bouring sequences intact. The 2.5 kb rrnX fragment was digested with BsuRI which yields a 747 bp fragment, containing the tandem promoters of the rrnX operon (Fig. 1). A 660 bp fragment which covers about the corresponding region with the rrnE promoters was derived from the 1.4 kb rrnE fragment by AluI digestion (Fig. 1). When these fragments were compared in the filter-binding assay the faster binding of the rrnX promoter fragment appeared to have been maintained (Fig. 2B). This observation makes it very likely that the differences are due to the ribosomal RNA promoters proper. Since both operons only differ beyond 20 basepairs upstream from the first transcription initiation site P_1, sequences in this region must be involved in promoter functioning, and cause the differences observed.

Heparin sensitivity of RNA polymerase-rRNA promoter complexes.

A very striking difference between the rrnX promoter and the rrnE promoter is heparin sensitivity of their complexes with RNA polymerase. In the experiments shown in Fig. 3, complexes were formed for five minutes between RNA polymerase and the 2.5 kb rrnX and the 1.4 kb rrnE fragment, respectively, and incubated with single stranded (ss) DNA for five minutes (see Materials and Methods of Ref. 6) to get rid of the unstable complexes. Then heparin was added. The dissociation of the stable complexes was measured. The rrnX promoter complexes turned out to be virtually heparin-resistent, while rrnE promoter complexes were heparin-sensitive to a certain extent. About 40% of rrnE promoter complexes are heparin resistent even at much higher heparin concentrations.

Since the ribosomal RNA operons each contain two active promoters (11,13) in tandem, which are both able to form stable complexes (6), a possible explanation for the heparin effects might be that one of the promoters of the rrnE operon is sensitive, whereas both promoters of the rrnX operon are resistent to heparin. This model includes that there are fragments on which only one of the rrnE promoters is used.

To investigate this matter we discriminated between the first and the second promoter of the rrnE operon using their different start sequence A,U and C,U, respectively. In the presence

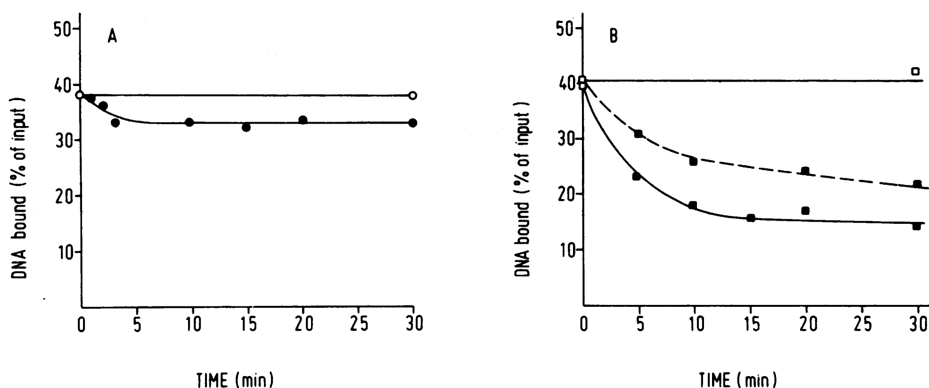


Figure 3. Heparin sensitivity of stable promoter complexes.
RNA polymerase (5.8 pmol/assay) was incubated with ^{32}P-end-labeled DNA fragments, under assay conditions which are described in Materials and Methods.
Panel A: Stable complexes formed with the 2.5 kb rrnX fragment (0.02 pmol/assay, spec. act. 273 Ci/mmol) were treated with heparin at 55 µg/ml (—●—) for the periods indicated. Panel B: Stable complexes formed with the 1.4 kb rrnE fragment (0.04 pmol/assay, spec. act. 50 Ci/mmol) were treated with heparin at 13 µg/ml (---■---) or at 55 µg/ml(—■—) for the periods indicated. Open symbols are incubations without heparin.

of ATP and UTP, the DNA will be trapped in a heparin-resistent transcription complex due to initiation on P_1. Table II shows that addition of ATP plus UTP, suppresses heparin-sensitivity of the rrnE promoter complexes completely, which indicates the first promoter of the rrnE operon as being heparin-sensitive. Simultaneous addition of CTP and UTP cannot fully prevent the dissociation caused by heparin. Complexes with the second rrnE promoter therefore appear to possess low heparin-sensitivity. Single nucleotides do not prevent heparin-induced dissociation at all in the case of CTP, and partially in the case of ATP and UTP. Possibly, these intermediate effects are caused by cross-contamination of the nucleotides ATP and UTP. Simultaneous addition of CTP and UTP suppresses heparin sensitivity about to the same extent as does the addition of UTP alone; thus the second rrnE promoter complexes may even be entirely heparin resistent.

The conclusion that can then be drawn from these experiments is that the heparin-sensitivity resides in the first promoter of

Table II. The effect of heparin in the absence or presence of nucleoside triphosphates, on stable rrnE promoter complexes.

Additions after stable complex formation				DNA bound
Heparin	A (mM)	U (mM)	C (mM)	(% of input)
-	-	-	-	40
+	-	-	-	23
+	0.4	0.2	0.2	41
+	0.4	0.2	-	41
+	-	0.2	0.4	30
+	0.4	-	-	29
+	-	0.4	-	28
+	-	-	0.4	23
+	0.2	0.004	-	27

RNA polymerase (5.8 pmol/assay) was incubated with the ^{32}P-end-labeled 1.4 kb rrnE fragment (0.04 pmol/assay, spec. act. 50 Ci/mmol) under the assay conditions described in Materials and Methods. Stable complexes formed, were treated with heparin (final concentration 55 µg/ml) or heparin plus various combinations of nucleotides for 15 minutes.

the rrnE operon and that the second rrnE promoter is heparin-resistent as are both rrnX promoters. This conclusion is in agreement with the fact that the sequences around P_2 in both operons are identical and the sequences of the first promoter regions of the rrnE and rrnX operon are different beyond 20 bp upstream from P_1 (10,11).

The mechanism of action of ppGpp.

Earlier we have reported that ppGpp decreases the stable complex formation between RNA polymerase and the 1.4 kb rrnE fragment (6). Stable complex formation between RNA polymerase and the 2.5 kb rrnX fragment is also inhibited by ppGpp (data not shown).

To investigate the mechanism of ppGpp action further we examined the complex formation between RNA polymerase and a 750 bp AluI fragment, containing the rrnE promoters (Fig. 1) in the absence and presence of ppGpp. Complexes formed were challenged with single stranded calf thymus DNA which traps free RNA

polymerase molecules. Because of rapid dissociation of loose complexes, their concentration will soon drop to zero upon incubation with ssDNA. The promoter complexes formed in the absence of ppGpp are of two kinds (6,30): a rapidly dissociating one, called C_I (closed complex) and a stable one, C_{II} (open complex). The concentration of complexes formed in the presence of ppGpp drops to zero upon incubation with ssDNA, albeit at a somewhat slower rate than the concentration of C_I (Fig. 4). When ln (DNA bound) is plotted versus time, straight lines are obtained; we calculate a first order decay constant of about 0.08 min^{-1} for complexes formed in the presence of ppGpp, which corresponds with a half-life of about 9 min. Similar data were obtained for the 747 bp BsuRI fragment, containing the rrnX promoters: a first order decay constant of about 0.06 min^{-1}, corresponding with a half-life of about 12 min. Since complexes formed in the presence of ppGpp are different from C_I as well as from C_{II} they are designated C_I'.

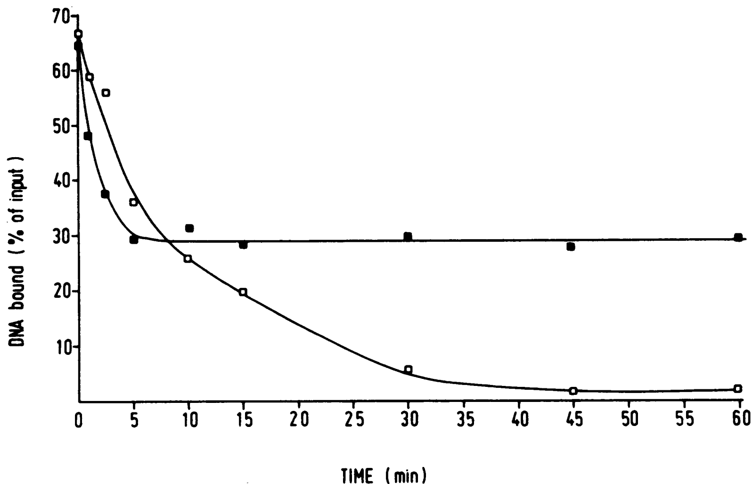


Figure 4. Decay of complexes formed in the absence or presence of ppGpp.

RNA polymerase (5.8 pmol/assay) was incubated with the ^{32}P-end-labeled 750 bp rrnE fragment (0.03 pmol/assay, spec. act. 91 Ci/mmol) for 15 minutes under standard assay conditions (40 mM KCl) in the presence (open squares) or absence (closed squares) of ppGpp (0.9 mM). Then one volume of warm binding buffer (with 40 mM KCl), containing ssDNA (final concentration 46 µg/ml) was added and incubation was continued for the periods indicated.

We wondered whether the unstable complexes, C_I and C_I' can be converted into stable transcription complexes by addition of the triphosphates ATP, UTP and CTP; this would prevent C_I and C_I' from decay during incubation with ssDNA, which was added simultaneously with the nucleoside triphosphates. When after complex formation in the absence of ppGpp the nucleotides and ssDNA are added simultaneously about the same amount of DNA as percentage of the input is bound to the filter as is without the addition of ssDNA and nucleotides (Table III, Fig. 4). Obviously the unstable complexes formed in the absence of ppGpp can initiate under these conditions. Initiation is specific for the start nucleotides of the tandem promoters of the rrnE operon, and does not occur when ssDNA, UTP and GTP are added simultaneously after complex formation. This indicates that the unstable complexes are largely productive in rRNA chain initiation and are not due to aspecific binding. From the heparin experiments we conclude that at least the heparin-sensitive part of stable rrnE promoter complexes can initiate too. We do not know whether initiation of C_I goes via C_{II}, or via an intermediate complex, common for initiation of C_I and C_{II}. Table III further shows that complexes formed in the presence of ppGpp are unable to initiate upon addition of the start nucleotides of the two rrnE promoters, but decay during

Table III. The effect of ppGpp and GDP on complex formation.

	Complex formation (% of DNA input) in the presence of		
	-	ppGpp	GDP
Additions after complex formation:			
-	64	67	-
ssDNA	29	5	33
ssDNA, A, U, C	60	4	60
ssDNA, U, G	33	-	-

Complex formation was carried out as described in the legends of Fig. 4 in the absence or presence of ppGpp (0.9 mM) or GDP (0.9 mM). After complex formation one volume of warm binding buffer (with 40 mM KCl) was added containing ssDNA (final concentration 46 μg/ml), either alone or in presence of the nucleoside triphosphates, indicated (concentration of ATP was 0.4 mM, of UTP, CTP, GTP 0.2 mM), and incubation was continued for 30 minutes.

incubation with ssDNA which is added simultaneously with the triphosphates. C_I' is therefore considered to be non-productive in rRNA chain initiation. The effect is specific for ppGpp and can not be obtained with GDP.

Using these very discriminatory conditions we determined the concentration of ppGpp needed for half maximal inhibition of productive complex formation. Figure 5 shows that this inhibition is obtained at 0.1 mM ppGpp, which corresponds very well with the K_i of ppGpp for rRNA accumulation in vivo (29). The same value was found with the 747 bp BsuRI fragment, containing the rrnX promoters (data not shown).

The next question concerned the effect of ppGpp on complexes formed in its absence. From the data presented in Table IV we conclude that C_I can be trapped as a transcription complex even in the presence of ppGpp. However, ppGpp given prior to the nucleotides for only 2 minutes suffices to turn over C_I into a

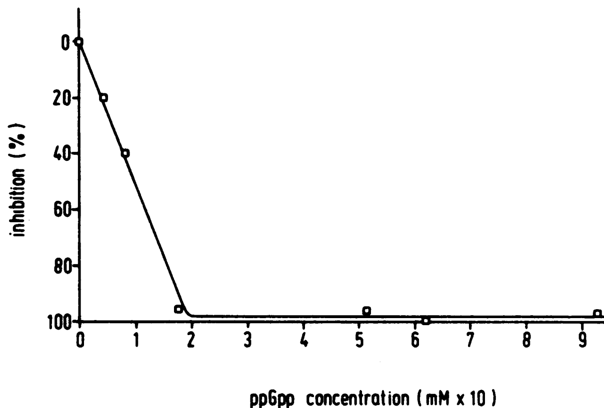


Figure 5. Concentration dependence of ppGpp inhibition.
RNA polymerase (5.8 pmol/assay) was incubated with the ^{32}P-end-labeled 750 bp rrnE fragment (0.03 pmol/assay, spec. act. 77 Ci/mmol) for 15 minutes under standard assay conditions (40 mM KCl) in the presence of ppGpp at the concentrations indicated. Then one volume of warm binding buffer (with 40 mM KCl) containing ssDNA (final concentration 46 µg/ml), ATP (0.4 mM), UTP (0.2 mM) and CTP (0.2 mM) was added and incubation was continued for 30 minutes. Further treatment was as described in Materials and Methods. DNA bound (% of input) in the control (incubation without ppGpp) was taken as 100%.

Table IV. The effect of ppGpp after complex formation.

Additions after complex formation	DNA bound (% of input)
ssDNA	30
ssDNA, ppGpp	23
ssDNA, GDP	30
ssDNA, A, U, C	58
ssDNA, A, U, C, ppGpp	62
ppGpp and after 2 min: ssDNA, A, U, C	26
ppGpp and after 15 min: ssDNA, A, U, C	26

Complex formation was carried out in the absence of ppGpp as described in the legends of Figure 5. After complex formation various combinations of compounds were added and incubation was continued for 30 minutes. Concentrations were as described in the legends of Table III.

non-productive form. The stable complex C_{II} seems to be dissociated by ppGpp only to a small degree.

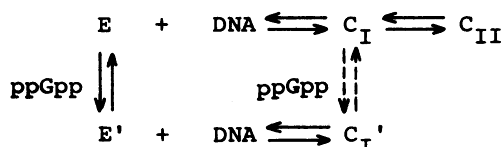
GENERAL DISCUSSION

The studies described in this paper reveal quantitative differences in RNA polymerase binding between the promoters of the rrnX and rrnE operon. Stable rrnX promoter complexes are formed faster than those of rrnE, and are more resistent to high salt and heparin. Transcription studies showed the same trend: RNA synthesis on rrnX is higher and less salt-sensitive than on rrnE. From the similar behavior of both parameters we may however not conclude that the rate of stable promoter complex (C_{II}) formation determines promoter strength, since initiation also proceeds fast from complex I (unstable complex). This conclusion follows from the observation that the initiation of C_I, brought about by the addition of the start nucleotides, succesfully competes with the decay, when C_I is challenged with single stranded DNA. Therefore, the rate of C_I formation should also be considered when the strength of different promoters is compared.

The nucleotide sequences of rrnX and rrnE are virtually identical over their entire transcribed region plus 20 basepairs preceding the first start site, P_1. Therefore, any difference observed in promoter functioning must reside in sequence differ-

ences upstream from this region and, obviously, be attributed to the first promoter. Indeed, we could pinpoint the difference in heparin sensitivity between rrnX and rrnE to the first promoter. When the nucleotide sequences beyond 20 bp upstream from P_1 are compared many differences between rrnX and rrnE emerge (12). The influence of the various sequences on the characteristics of promoter complex formation still has to be determined.

The specific inhibitor of rRNA synthesis, ppGpp, completely abolishes stable complex formation, but still allows unstable complexes to be formed. Since the unstable complexes formed in the presence of ppGpp have a significant longer half life than the normal unstable complexes (C_I), they are designated C_I'. These different half lifes will have caused an underestimation of the ppGpp effect in earlier experiments (6), since the 5 min challenge with ssDNA used there, is not sufficient to let all the C_I' complexes decay. Therefore, the degree of inhibition by ppGpp measured, will have depended on the extent of stable com-complex formation in the absence of ppGpp. In the present studies this problem was circumvented by allowing the unstable complexes to decay in the presence of ssDNA for longer periods of time. To indicate the relationship supposed between the various complexes found we used the following scheme:



Complexes formed in the presence of ppGpp (C_I') can not be trapped in stable transcription complexes by addition of the start nucleotides (Table III). This observation implies first that C_I' as such is non-productive, and second that C_I' can not enter into a stable transcription complex via the dissociation step to C_I. Therefore the decay of C_I' which becomes visible when free RNA polymerase (E) and/or RNA polymerase/ppGpp complex (E') are removed by ssDNA, must preferably follow the lower route of our scheme. A similar reasoning holds for the formation of C_I'. If the route were via complex I, DNA could be prevented from entering into C_I' by adding the nucleotides for initiation.

Again this does not happen, whereas initiation from complex I, accumulated in the absence of ppGpp, succesfully competes not only with C_I decay, but also with C_I inactivation by ppGpp (Table IV).

While ppGpp inhibits rRNA synthesis, it stimulates the transcription of a number of other operons, for example the lac operon (31). We feel that the scheme we have proposed could still be valid in these cases. Instead of being non-productive, as with rRNA promoters, the ppGpp-containing complex C_I' could be more effective in initiation than C_I in the case of these other promoters.

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REFERENCES

1. Cashel, M. and Gallant, J. (1969) Nature 221, 838-840.
2. Ooyen, A.J.J. van, de Boer, H.A., AB, G. and Gruber, M. (1975) Nature 254, 530-531.
3. Ooyen, A.J.J. van, Gruber, M. and Jørgensen, P. (1976) Cell 8, 123-128.
4. Reiness, G., Yang, H.-L., Zubay, G. and Cashel, M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2881-2885.
5. Oostra, B.A., Ooyen, A.J.J. van, and Gruber, M. (1977) Molec. Gen. Genet. 152, 1-6.
6. Hamming, J., Gruber, M. and AB, G. (1979) Nucl. Acid. Res. 7, 1019-1033.
7. Kenerley, M.E., Morgan, E.A., Post. L., Lindahl, L. and Nomura, M. (1977) J. Bacteriol. 132, 931-949.
8. Kiss, A., Sain, B. and Venetianer, P. (1977) FEBS Lett. 79, 77-79.
9. Boros, I., Kiss, A. and Venetianer, P. (1979) Nucl. Acid. Res. 6, 1817-1830.
10. de Boer, H.A., Gilbert, S.C. and Nomura, M. (1979) Cell 17, 201-209.
11. Young, R.A. and Steitz, J.A. (1979) Cell 17, 225-234.

12. Csordas-Toth, E., Boros, I. and Venetianer, P. (1979) Nucl. Acid. Res. 7, 2189-2197.
13. Gilbert, S.C., de Boer, H.A. and Nomura, M. (1979) Cell 17, 211-225.
14. Glaser, G. and Cashel, M. (1979) Cell 16, 111-121.
15. Jørgensen, P. and Fiil, N.P. (1976) in Control of Ribosome Synthesis, Kjeldgaard, N.O. and Maaløe, O., Eds. pp. 370-382, Copenhagen Munksgaard.
16. Travers, A.A. (1980) J. Bacteriol. 141, 973-976.
17. Yamamoto, M. and Nomura, M. (1976) FEBS Lett. 72, 256-261.
18. Greene, P.J., Heyneker, H.L., Bolivar, F., Rodriguez, R.L., Betlach, M.C., Covarrubias, A.A., Backman, K., Russel, D.J., Tait, R., Boyer, H.W. (1978) Nucl. Acid. Res. 5, 2373-2379.
19. Baksi, K., Rogerson, D.L., Rushizky, G.W. (1978) Biochemistry 17, 4136-4139.
20. Burgess, R.R. and Jendrisak, J.J. (1975) Biochemistry 14, 4634-4638.
21. Cashel, M. (1974) Anal. Biochem. 57, 100-107.
22. Miller, J.H. (1972) in Experiments in Molecular Genetics, pp. 321-325 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
23. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
24. Seeburg, P.H., Nüsslein, C. and Schaller, H. (1977) Eur. J. Biochem. 74, 107-113.
25. Young, R.A., Macklis, R. and Steitz, J.A. (1979) J. Biol. Chem. 254, 3264-3271.
26. Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. (1978) Proc. Natl. Acad. Sci. USA 4801-4805.
27. Jørgensen, P., Collins, J., Fiil, N., van Meyenburg, K. (1978) Molec. Gen. Genet. 163, 223-228.
28. Carbon, P., Ehresmann, C., Ehresmann, B. and Ebel, J.P. (1978) FEBS Lett. 94, 152-156.
29. Fiil, N.P., Von Meyenburg, K. and Friesen, J.D. (1972) J. Mol. Biol. 71, 769-783.
30. Chamberlin, M. (1976) in RNA polymerase, Losick, R. and Chamberlin, M., Eds. pp. 17-67, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
31. Primakoff, P. and Artz, S.W. (1979) Proc. Natl. Acad. Sci. USA 76, 1726-1730.