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Ribosome associated protein(s) specifically bind(s) to the upstream activator sequence of the *E. coli rrn*A P1 promoter

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ABSTRACT

A sequence located upstream to the *E. coli rrn*A P1 promoter is required for optimal promoter activity. Deletion of this sequence reduces *in vivo* transcription by 90%. Substitution of this upstream activating sequence with the unrelated bent DNA sequence of the kinetoplast of *Crithidia fasciculata* , restores *in vivo* expression to high levels . Cellular proteins which are present only in exponentially growing cells bind specifically to intact *rrn*A P1, but do not bind to the promoter missing the upstream activating sequence. These proteins are associated with the 30S ribosomal subunits but can be washed off with concentrated salt. The correlation between the binding activity and cell growth rate suggests a role for these proteins in the transcriptional control of rRNA synthesis.

INTRODUCTION

E. coli 's ability to respond to amino acid starvation (stringent control) or to nutritional conditions which limit growth rate (growth control) by dramatically decreasing expression of stable RNA genes has been the subject of extensive studies (1,2). Thus, it has been established that expression of rRNA genes is optimal in exponentially growing cells.

Sequences located upstream of promoters of stable RNA genes have been shown to be important for their expression. Using promoter-*lacZ* fusions, Gourse *et al.* (3) have demonstrated that deletion of the sequence between −51 and −88 relative to the transcription start site of the *rrn*B P1 promoter decreases transcription by at least 15-fold. Lamond and Travers (4) have shown that deletion of the sequence between −40 and −98 decreases transcription from the *tyr*T tRNA promoter by 10- to 12-fold. Similarly, Bossi and Smith have shown that deletion of 3 base pairs at −70 of the *his*R tRNA gene in *Salmonella typhimurium* decreases transcription by 60% (5). Thus, in each of the stable RNA promoters studied to date, a distinct sequence upstream to the −35 sequence of the canonical promoter is responsible for the high level of expression. This sequence has been called the upstream activator sequence (UAS)(3).

Two regions, the −10 (Pribnow box) and the −35 sequence are conserved among prokaryotic promoters. *E. Coli* operons that are known to be regulated by the same elements, contain additional homologous DNA sequences in their promoter region (6,7). Thus, one would expect to find common structural or sequence elements in the promoter region of stable RNA genes as well. A comparison of the the upstream regions of a large number of rRNA, tRNA, and ribosomal protein genes revealed no overall sequence homology (8). However, there are some indications that a common DNA conformational feature exists in the upstream region of these promoters (9). Bossi and Smith have demonstrated that a fragment carrying the upstream activator sequence (UAS) of the *his*R gene exhibits abnormal electrophoretic mobility; at 65°C its mobility is close to normal for its size,

but between 5°C and 37°C its mobility is increasingly retarded. Moreover, the same 3-bp deletion at −70 which reduces transcription by 60% also restores normal electrophoretic mobility to the fragment at low tempratures (5). Gourse *et al.* have indicated that fragments carrying the UAS of *rrn*B run approximately 25% more slowly in polyacrylamide gels than expected from their length (3). Such abnormal electrophoretic behaviour of DNA fragments in an acrylamide gel has been interpreted as reflecting perturbations in the DNA structure, particularly a bend or kink (10). The UAS of *rrnA P1 h*as been shown to be recognized by an unique enzyme from trypanosomatids that recognizes bent DNA structures (11), hinting that this UAS has similar conformational characteristics as rrnB.

In this work we demonstrate that a sequence upstream of the *E. coli rrn*A P1 promoter is required for maximal promoter activity. Deletion of this upstream sequence reduced transcription activity *in vivo* by 90%. Substitution of this UAS with the unrelated Kinetoplast DNA sequence of *Crithidia fasciculata*, that is known to be intrinsically bent, restored the high levels of expression *in vivo*. We also show that cellular proteins which are present only in exponentially growing cells bind specifically to the *rrn*A P1 promoter region, but do not bind to the promoter that is missing the UAS. These protein(s) sediment together with the 30S ribosomal subunit fraction but can be further purified by washing these 30S ribosomal subunits with concentrated salt.

MATERIALS AND METHODS

Plasmids and bacterial strains

Plasmid pGC96 was obtained from Dr. M. Cashel. This plasmid contains an intact *rrn*A P1 fragment from −262 to +32 around the transcription start site. This plasmid was derived from pES3 (12), so that in pGC96 the IS2 terminator was replaced by λtRI terminator. As described in Fig. 1a, a Box A region is present downstream from the promoter. The *rrn*A P1 promoter region in pGC96 is cloned between EcoRI and SalI sites. Plasmids pGC96D and pGC96DC were derived from pGC96 by deleting the −262 to −47 region of *rrn*A P1, leaving only −47 to +32. In plasmid pGC96DC the −262 to −47 region was replaced with the 210 bp fragment of the kinetoplast of *Crithidia fasciculata* . This fragment is a StuI-AccI fragment from plasmid pPK201, obtained from Dr. J. Shlomai (13).

The host cell used with these plasmids, and for protein extraction was *E. coli* CF 748, a relA$^+$ strain obtained from Dr. M. Cashel (14).

RNA preparation and primer extension

RNA isolation was carried out according to Sarmientos *et al.* (14). The cells were grown at 37°C in LB medium (15) to mid log phase (OD_{600nm} of 0.2−0.3). 2ml aliquots were added to a mixture containing 0.5ml of 1M NaCl, 2.5% SDS and 50mM EDTA (pH 8) and boiled for 10−15 sec. After phenol extraction and ethanol precipitation the RNA was dissolved in 0.4ml of 0.3M sodium acetate (pH 5) and used for primer extension analysis. The primer extension reaction (16) was performed with the modifications described previously (17). The total RNA preparation from the 2ml culture was mixed with 8 ng of 5′-^{32}P labeled oligonucleotide DNA primer and precipitated with ethanol. The precipitate was resuspended in 45μl of 100mM Tris-HCl (pH 8.3), 140mM KCl and 10mM $MgCl_2$. The mixture was incubated at 65°C for 10 min and was cooled at room temperature for 5 min. Dithiothreitol was added to a concentration of 10mM and the four dNTPs to 1mM each. 15 units of AMV reverse transcriptase were added and the mixture was incubated at 42°C for 1 hr. The reaction was stopped at 75°C for 10 min. 1μg of

RNase was added and incubation continued for 30 min at 37°C followed by ethanol precipitation. The primer sequence was 5'-GAGCAGTGCCGCTTCGC-3', complimentary to the Box A sequence downstream to the rRNA promoters in the plasmids described above (12 and Fig 1a.). The primer extension products were quantitated using the GS300 Scanning Densitometer, Hoeffer Scientific Instruments (San Francisco).

DNA preparation and end-labelling for gel retardation assay

The plasmids carrying *rrn*A P1 were digested with EcoRI and SalI and end-labeled with *E. coli* DNA polymerase Klenow fragments and α-^{32}P dATP for 20 minutes at room temperature (15).

Protein extract

A crude preparation of cellular protein was prepared by a modification of the method described by Nash and Robertson (18). *E. coli* cells were grown in LB medium at 37°C. At the desired growth phase the cells were spun down at 4°C for 10 min and then quick-frozen in liquid nitrogen. After thawing the cells were resuspended in 2 volumes of ice-cold 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20 mM NaCl, and 10% (w/v) glycerol. The cells were disrupted on an ice-cold Yeda-press (Linca, lamon Instrumentation Co. LTD) for two periods of 10 min with 5 min in between. After lysis the suspension was centrifuged for 40 minutes at 30000g at 4°C, and the supernatant was dialyzed overnight at 4°C against a buffer consisting of 10 mM Hepes (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 50% (v/v) glycerol. The resulting crude protein extract was divided into aliquots and stored at −70°C. The protein concentration was determined by the method of Bradford (19).

Ribosome preparation

A crude ribosome extract was prepared by the method of Block and Haseltine (20) from mid-log culture (OD_{600}=0.55) grown in 1 liter LB medium at 37°C. The cells were harvested by centrifugation and resuspended in 10mM MgOAc., 100mM Tris Ac.(pH 8) and 1 mM DTT to an OD_{600}=40. Lysozyme was added to 1 mg/ml. After lysis by two periods of 10 min on Yeda-Press, 5μg/ml RNase free DNase was added. Ribosomal buffer (50mM Tris Ac.(pH8), 1 mM DTT, 10mM MgOAc., 27mM Ammonium Ac.) was added to a final volume of 120 ml, and spun at 30000g for 20 min. The supernatant was centrifuged at 100000g for 3 hrs and the resulting pellet was resuspended in 2 ml of ribosomal buffer, and stored at −70°C. 70S, 50S and 30S ribosomal subunits were prepared according to Eikeberry et al. (21).

High salt washed ribosomes were prepared by increasing the KCl concentration to 1M, stirring for 1 hr in ice, and respinning. The pellet was resuspended in ribosomal buffer and stored at −70°C.

Binding conditions and gel retardation

We used the gel retardation method of Garner and Revzin (22) to detect protein-DNA complexes by their retarded mobility in acrylamide gels relative to naked DNA. Crude cellular protein extract, ribosome extract or ribosomal subunits were added, as indicated, to a final volume of 10 μl of binding mixture. The binding mixture contained 40 ng end-labeled DNA,1.5μg poly(dI-dC), 50mM Tris HCl(pH 7.4) 70 mM KCl, 15 mM NaCl, 10 mM β-mercaptoethanol and 1mM EDTA. The mixtures were incubated at 25°C for 20 min and 4μl of loading buffer (0.1% xylene cyanol and 0.1% bromophenolblue in 50% glycerol) were added. The samples were loaded on a 5% polyacrylamide gel in 0.5% TBE buffer and run at 10 V/cm. The gels were dried and subjected to autoradiography.

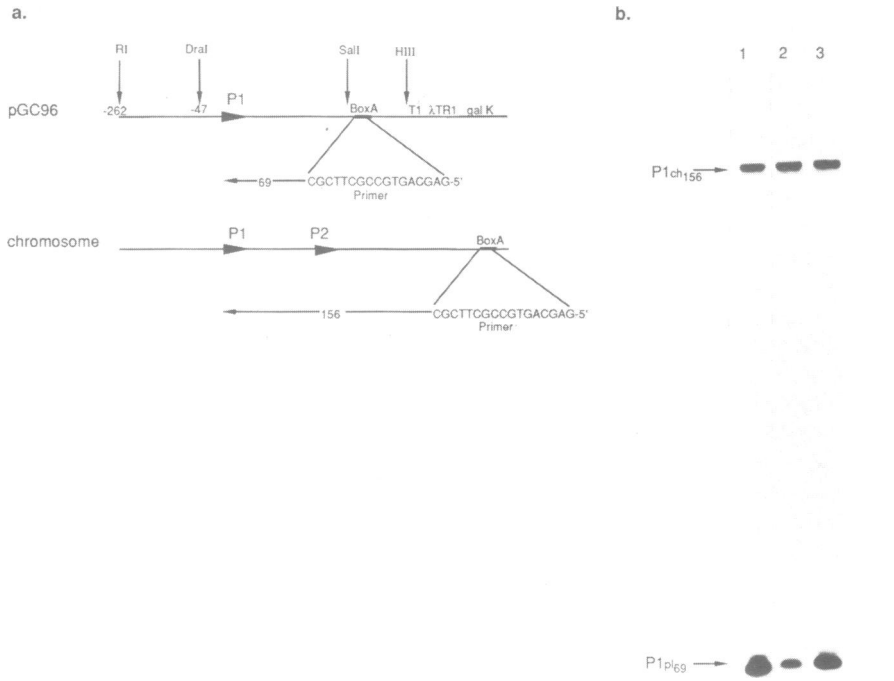

Figure 1 Transcription from *rrn*A P1 is affected by sequence changes upstream to the canonical promoter. (a) A schematic representation of the promoter regions in the chromosomal P1 and in the different plasmids that carry P1 promoter (not drawn to scale). The primer that was used was complimentary to the Box A sequence, thus two sizes of cDNA were seen in each RNA sample,which correspond to chromosomal born RNA and plasmid born RNA. The physical location of the restriction sites described in the text, the Box A sequence and the terminators are schematically represented in the figure. RI = EcoRI, HIII = HindIII.
(b) cDNA synthesis. RNA was extracted from logarithmically growing cells (OD_{600}=0.25) and was subjected to primer extension analysis using a DNA primer complimentary to the Box A sequence located downstream from the transcription start point. The resulting cDNA synthesised of the chromosomal or plasmid RNA was 156bp and 69bp respectively. Lane 1—RNA from cells harboring pGC96, which contain the intact wild-type *rrn*A P1 promoter. Lane 2—RNA from cells harboring pGC96D in which the EcoRI-DraI segment upstream to P1 has been deleted (see a). Lane 3—RNA from cells harboring pGC96DC, in which the original EcoRI-DraI segment has been replaced by a 210-bp bent DNA fragment from *Crithidia fasciculata* kinetoplast (13). The transcription start sites were identified by running dideoxy sequencing reactions in parallel, using the same primer (not shown); these correspond to the previously published start sites (14).

RESULTS

Insertion of an unrelated bent DNA fragment upstream of a deleted rrnA P1 promoter restores high level promoter activity

The finding that upstream sequences are important for rrnB P1 promoter activity, and that these sequences show *in vitro* characteristics of bent DNA (3), prompted us to study the importance of the DNA conformation in the region upstream of canonical sequence of the rrnA P1 promoter. We reasoned that if the conformation of the DNA at this region

is of importance for its ability to activate this promoter, we might be able to replace it by an unrelated DNA sequence which is also bent.

To examine the importance of the rrnA P1 upstream region and the effect of its conformation on the promoter activity we constructed the following plasmids: pGC96 containing the wild type P1 (fig.1a), pGC96D containing P1 missing the upstream −47 to −262 sequence, between the DraI and EcoRI sites, and pGC96DC in which the deleted upstream region was replaced by the bent kinetoplast DNA of *Crithidia fasciculata* .

We used the primer extension technique to study the *in vivo* level of transcription of the different *rrn*A P1 constructs. A primer complementary to the Box A sequence (12) was annealed to RNA extracted from exponentially growing cells carrying the different plasmid constructs. A complimentary DNA was then synthesized by reverse transcriptase. As diagrammed in Figure 1a, the intact chromosomal *rrn* operons contain additional DNA sequences of the second promoter P2, between P1 and Box A, which are not present in our plasmid constructs. Thus, one can easily differentiate between RNA transcribed from the chromosomal genes or from the plasmids by the length of the reverse transcripts. As indicated in the figure, the reverse transcriptase product corresponding to the chromosomal P1 transcript is 156 bases long while the plasmid-derived RNA gives a 69 bases long product. Furthermore, the constant level of the chromosomal transcript provides an internal control for the efficiency of RNA extraction from the cells, and enable us to compare quantitative differences in promoter activity between the various plasmids. The copy number of the different plasmids was found to be the same. It should be noted that transcription from chromosomal P2 could not be detected under these conditions, as the primer used was too close to the P2 start site, reverse transcript of this RNA should give rise to 30 bases long transcript that would not be detected under the experimental conditions used.

Lanes 1 and 2 in Figure 1b demonstrate that an EcoRI-DraI deletion of the UAS (from −262 to −47) resulted in a 90% reduction of transcription initiation in exponentially growing cells (as measured by density scanning of the X-ray film). This is in agreement with previously described results for *rrn*B (3). Lane 3 demonstrates that substitution of the EcoRI-DraI UAS region by a DNA fragment from the kinetoplast of *Crithidia fasciculata* restored the high-level activity of the promoter, thus in the presence of this insert the activity of the promoter was 70% of that of the wild type promoter (Fig 1b Lane 3). This kinetoplast DNA fragment from *Crithidia* has been shown to be highly curved (23). Thus, P1 activity is probably sensitive to changes in the DNA conformation in the region upstream to the −35 region. Furthermore it appears likely that the curvature of the UAS region is an important factor in the ability of this sequence to promote high levels of P1 transcription.

Cellular proteins present only in exponentially growing cells bind specifically to rrnA P1 promoter region

Since rRNA P1 promoter activity depends largly on the bacterial growth rate, one would expect to find changes in the activity of regulatory factors under different growth conditions. In the previous section we have demonstrated the importance of the UAS for optimal expression of *rrn*A P1. The region upstream of promoters has been shown in many cases to be important for promoter regulation and to be the target for binding of regulatory proteins. We therefore thought that the UAS is a likely candidate to serve as a binding target for P1 regulatory factors.

We used the gel retardation technique to search for protein factor(s) which bind specifically to the upstream activator sequence of the *rrn*A P1 promoter. Gel retardation detects protein-

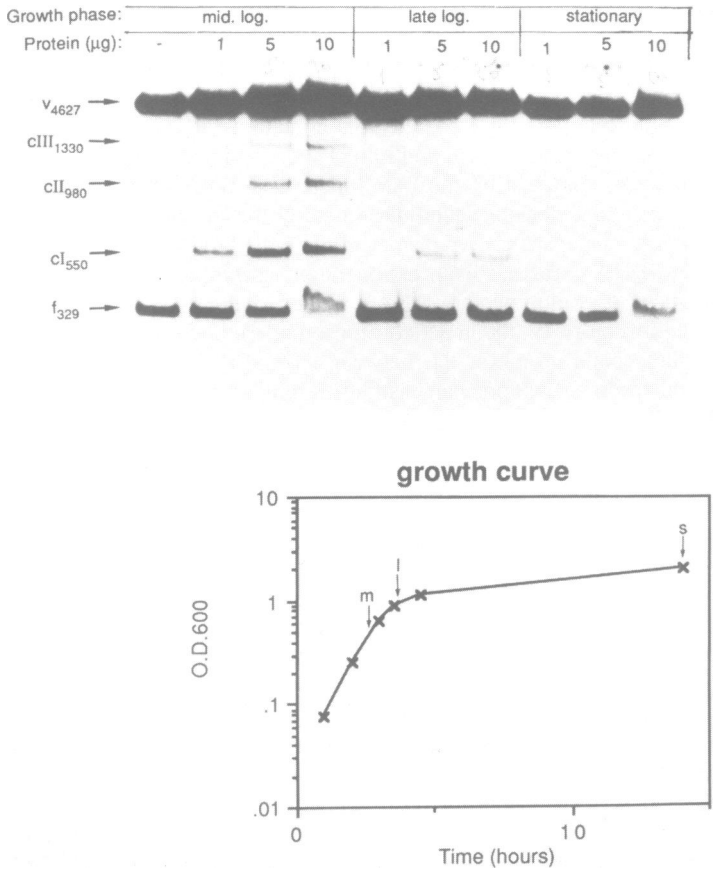

Figure 2 Gel retardation analysis of *rrn*A P1 promoter fragment with crude cell extracts. Plasmid pGC96 was digested with EcoRI and SalI (see Fig 1a), end-labelled, and incubated with varying amounts of crude extracts from mid log (m), late log (l) and stationary (s) growth phases, as indicated. The protein-DNA complexes were run on an acrylamide gel as described in Methods. cI, cII and cIII indicate the three complexes formed by the fragment. DNA markers were run in parallel (not shown); the size of the complexes are indicated as DNA length in bp. The length of the vector (v) and the free fragment (f) were calculated from the sequence of the plasmid and the restriction sites.

DNA complexes by their retarded mobility on polyacrylamide gels relative to naked DNA (22). We prepared total cellular extracts from cell cultures at three growth phases: mid-logarithmic, late-logarithmic, and stationary phase. Plasmid pGC96 was digested so that the *rrn*A P1 promoter and its UAS were on the short fragment. The fragments were end-labelled and then incubated with increasing amounts of the three protein extracts, in the presence of fixed amount of poly (dI-dC), to avoid non-specific DNA binding. In the presence of the mid-log cell extract, the P1 fragment migrated in three descrete new bands termed: cI, cII and cIII (Figure 2, mid. log.). At the lowest protein concentration only the cI band was detected, while the cII and cIII bands became evident at higher extract concentrations. The mobility of the vector was not affected even at high protein extract

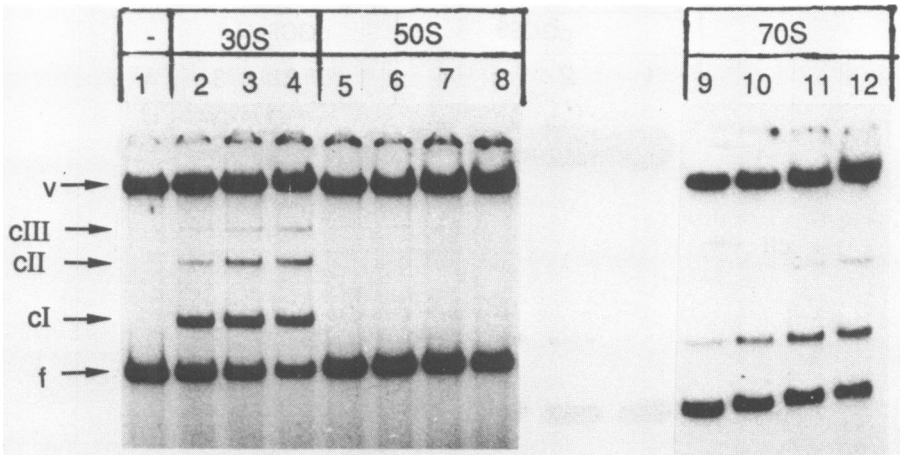

Figure 3 Ribosomal fractions bind to the *rrn*A P1 fragment. Plasmid pGC96 was digested with EcoRI and SalI, end labelled, and incubated with varying amounts of 70S ribosomes, 30S, or 50S ribosomal subunits fractions (see materials and methods). Lane 1: No ribosome. Lanes 2–4: 12.5, 25 and 50 pmoles of 30S ribosomal subunit, respectively. Lanes 5–8: 25, 40, 50, and 75 pmoles of 50S ribosomal subunit, respectively. Lanes 9–12: 10, 20, 50, and 100 pmoles of 70S ribosomes, respeectively. V- vector; f- P1 fragment; cI,cII,and cIII—the DNA–protein complexes.

concentrations. Gradually increasing the amount of poly(dI-dC), while keeping constant the amount of plasmid DNA and cell extract, resulted in the titration of the DNA-protein complexes in the same manner as observed with decreasing amounts of protein extract (Fig. 2,mid. log.); thus with the increase of poly (dI-dC), cIII disappeared first while cI was affected last (data not shown). These results indicate that the protein which forms the cI band has a high affinity for the *rrnA* P1 DNA fragment and/or is present in high abundance.

When a late-log protein extract was used for the DNA binding experiment, only the cI complex was formed and in very small amounts, even at the highest protein concentration (Fig.2, late log.). Stationary phase cell extract did not form any detectable DNA-protein complex (Fig. 2, stationary). These results reveal that at least one cellular protein binds to the *rrn*A P1 promoter region, and that the binding activity correlates well with the rate of cell growth.

We have also tested the effect of elevated levels of ppGpp on the binding activity found in the cell extract. High levels of ppGpp in the cells occur usually under nutritional stress (1). We obtained high ppGpp levels, and consequently slower growth rates without changing the nutritional conditions of the cells by the induction of a strain containing a relA ORF cloned under an inducible promoter (G. Glaser et.al. , manuscript in preparartion). The induction of this ORF caused an immediate cessation of cell growth concomitant with an abrupt burst of ppGpp level. However, a decrease in the ability of the induced cell extracts to bind to the P1 fragment, was observed only after changes in ppGpp levels and growth rate have already occured, suggesting that the effect of ppGpp on the binding activity may be indirect (data not shown).

In order to eliminate the possibility that the retardation of P1 fragment is due to RNA polymerase binding, we performed the gel retardation experiment using purified RNA

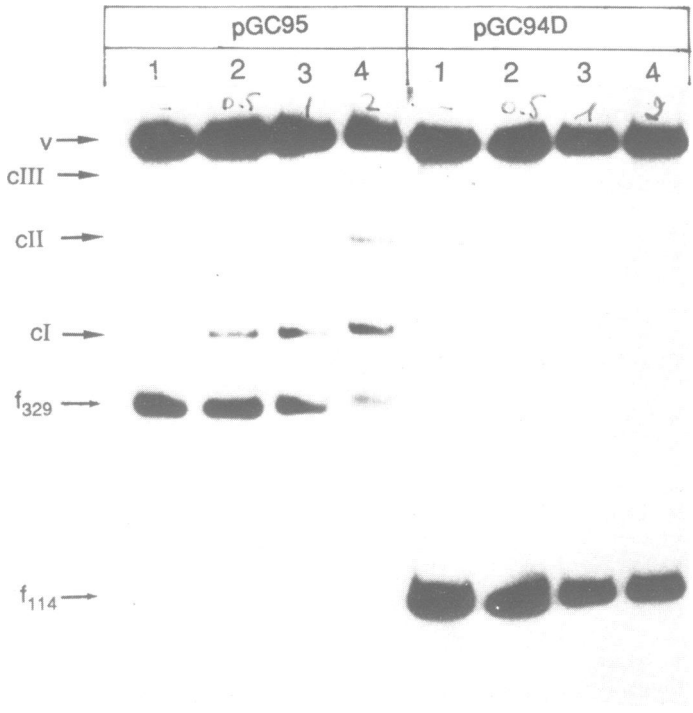

Figure 4 Truncated P1 promoter is unable to form DNA-protein complexes with the 30S ribosomal subunits fraction. Plasmid pGC96D, which contains a deletion from −262 to −47, and plasmid pGC96 containing the entire P1 sequence (see materials and methods), were digested with EcoRI and SalI, end- labelled and incubated with (1) 0, (2) 2.5 , (3) 25 and (4) 50 pmoles of 30S ribosomal subunits fraction. The intact and truncated P1 fragments are 329bp and 114 bp long respectively.

polymerase instead of the cell extract. No retardation of the P1 fragment was detected unless very large amounts of polymerase, exceeding by far the estimated physiologic concentration, were used. Under these conditions the fragment was retarded to a higher position of the gel then either cI, cII or cIII (data not shown). It therefore seems that RNA polymerase is not the factor involved in the formation of the cI, cII or cIII DNA-protein complexes.

The cellular proteins that bind to rrnA P1 are associated with the 30S ribosomal subunits

In an attempt to localize the cellular fraction that contains the binding activity, we have spun the cell extract at 100000g and separated the crude ribosome pellet from the supernatant. When these crude ribosomes (or pure 70S ribosomes) were used in the gel retardation assay, three complexes were formed with P1 and they migrated to the same position as seen previously with the total cellular extract (Figure 3, lanes 9−12 and Figure 5,crude ribosomes). No binding activity was observed in the supernatant of 100000g (S100).

When we examined the ability of isolated 30S or 50S ribosomal subunits to form complexes with P1, only the 30S fraction showed binding to the P1 fragment, forming the same three complexes as the crude ribosomes preparartion (Figure 3, lanes 1−8). Note

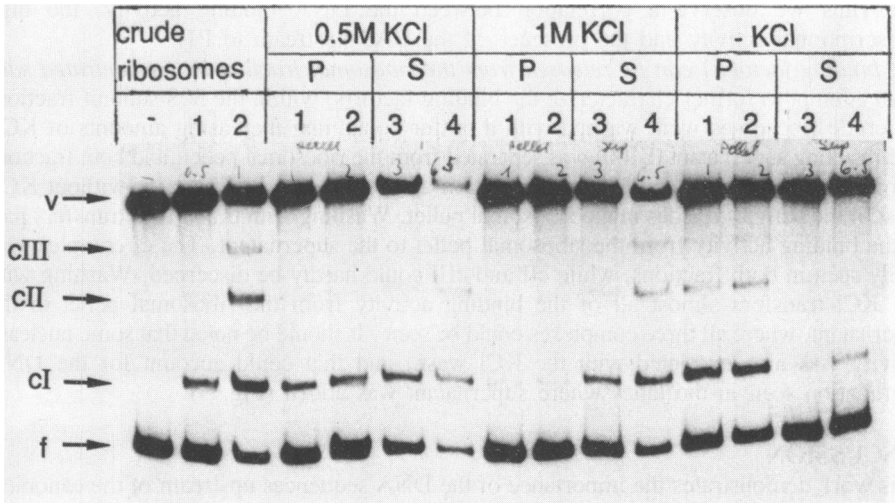

Figure 5 The binding activity can be removed from the ribosomal fraction by a KCl wash.
Crude ribosomes were washed with varying concentrations of KCl and the supernatant was separated from the pellet following centrifugation at 100000g. End labelled EcoRI−SalI fragments of pGC96 were incubated with varying amounts of either the pellet, (P), or the supernatant, (S), and run on a polyacrylamide gel. 1—1.2, 2—2.5, 3—0.6, 4—1.2 μg protein.

that the binding activity of the 30S subunits is higher than that of an equimolar amount of 70S ribosomes.

We have performed several experiments in order to determine the physical properties of the binding activity found in the cell extract. Heating the 30S subunit fraction to 65°C for 5 minutes prior to the binding experiment abolished the formation of the cII and cIII bands. Heating for an additional 3 minutes caused a decrease also in the amount of cI formed (not shown). Treatment with RNase T1 did not cause any reduction in complex formation relative to untreated 30S subunits; it even caused a slight increase in the binding activity. Treatment of the 30S subunit fraction with Proteinase K completely abolished their binding activity (not shown), indicating that the activity is due to proteins.

The P1 upstream activator sequence -UAS- is the target of the binding activity

Gel retardation experiments were performed using plasmid pGC96D which contains a deletion from −262 to −47 of P1. As seen in Figure 4, this truncated P1 fragment unlike the intact P1 was not retarded by the 30S ribosomal subunits. Furthermore, this deleted P1 fragment was unable to compete for binding with the non-deleted P1 fragment (data not shown). To prove that the sequences themselves and not merely the distance from the promoter are required for the binding of the 30S fraction, we examined the binding ability of the truncated P1 fused to random pBR322 sequences. Like the truncated P1, this full-length fused promoter did not form a DNA-protein complex with the 30S subunits fraction. We have also checked pGC96DC for binding cellular proteins. However, due to the fact that the fragment that carries P1 fused to the Kinetoplast bent DNA, migrated very little into the gel, one can hardly detect changes in gel migration due to other factors. Thus, we can not exclude binding of cellular proteins to this chimera fragment. As shown above (Fig.1b), the deleted P1 retains only 10% of the transcriptional activity of wild type

P1. Thus we observe a correlation between the DNA binding activity, the high transcriptional activity and the presence of the UAS upstream to P1.

The binding factor(s) can be released from the ribosomal fraction by concentrated salt

In an attempt to further characterize the binding factor(s) within the 30S subunit fraction, the crude ribosomes were washed with a buffer containing increasing amounts of KCl. The resulting supernatant (S100) was separated from the ribosomal pellet, and both fractions were tested for P1 binding by gel-retardation assay. As seen in Figure 5, without KCl, most of the activity resides in the ribosomal pellet. Washing with 0.5M KCl transfers part of the binding activity from the ribosomal pellet to the supernatant. The cI complex was easily seen in both fractions, while cII and cIII could hardly be discerned. Washing with 1M KCl transfers almost all of the binding activity from the ribosomal pellet to the supernatant, where all three complexes could be seen . It should be noted that some nuclease activity was also extracted with the KCl wash, and that could account for the DNA degradation seen in the lanes where supernatant was added (Fig. 5).

DISCUSSION

This work demonstrates the importance of the DNA sequences upstream of the canonical −10 to −35 region in the transcription activity of the *E. coli's rrn*A P1 promoter, and examines the ability of protein factors to bind specifically to this region.

Using the method of primer extension we have demonstrated that the UAS is required for maximal expression of ribosomal RNA P1 promoter *in vivo* . As was previously demonstrated for *rrn*B, we show here that P1 expression *in vivo* is reduced 90% by deleting the −267 to −47 region. Replacing this deleted region with the Kinetoplast DNA of *Crithidia fasciculata* , which is known to be bent, restores the promoter activity *in vivo* to nearly wild type levels. (Figure 1).

The UAS, of other stable RNA gene promoters, has been the focus of numerous investigations. Conformational changes in this region have been suggested based upon the interaction of RNA polymerase with the promoter region (24). As mentioned in the introduction, the abnormal electrophoretic mobility of fragments carrying the UAS of the *hisR* (5) and rrnB genes (3) has been interpreted as reflecting curvature or bending of the DNA. Although no sequence homology between the various stable RNA gene UAS's has been found, there may be homology at the DNA conformation level. It has been suggested before that one possible significance of DNA bending, is that the stored free energy could be used to perform mechanical work such as opening the DNA helix during transcription initiation (10,25,26). DNA bending could also bring remote sequences to close proximity with the promoter (10). We show here that bent DNA upstream to the P1 promoter affects strongly the expression of this promoter. These sequences may be the target, or recognition site for regulatory proteins. Our observations are only circumstantial and it still may be that other additional factors may be responcible for activation of rRNA promoters.

Using the method of gel retardation we have demonstrated the presence of three DNA-protein complexes when crude cellular extracts, crude ribosomes or 30S ribosomal subunits were incubated with P1 fragments which include UAS. The binding activity of these proteins correlates with the bacterial growth rate, suggesting involment in growth rate control of rRNA synthesis. Moreover, we have shown that DNA-protein complex formation requires the specific presence of the UAS, since no binding was seen when this sequence was deleted (Figure 4) or replaced by a pBR322 sequence of equal length, nor was binding activity

titrated out by the truncated P1 which lacks the UAS. We are not in a position as yet to state how many proteins are involved in the formation of the three DNA-protein complexes. The possibility that complexes cII and cIII are formed by adding additional molecules of the cI protein to the complex can not be ruled out. However, this possibility seems unlikely for two reasons: first, elevating extract concentration in the binding reaction increases the amount of cII and cIII formed without decreasing the amount of cI. Second, adding very large amounts of 30S ribosomal subunits lead to a reduction in the amount of cI and the appearance of a new complex more retarded than cIII, without any change in the amount of cII or cIII (not shown). If the three observed complexes represent in fact different proteins that bind to P1, then the protein that forms cI is either the most abundant, or has the highest affinity for UAS since cI is always the dominant complex seen on the gel.

A major observation in the present study is that the binding activity is associated exclusively with the 30S ribosomal subunit (Figure 3). In addition, this activity can be washed out from the ribosomes with 1M KCl, thereby transfering the binding activity that forms all three complexes, from the ribosomal pellet to the supernatant (Figure 5).

Our results lead also to the conclusion that the cell extract binding activity is enhanced when the protein(s) are not bound to ribosomes. Firstly, the binding specific activity is greater in the KCl-wash supernatant than in the crude ribosomes. Secondly, the fact that the binding activity of the 30S subunit is greater than that of an equimolar amount of 70S ribosomes (Figure 3) indicates that formation of 70S partially masks the UAS-binding proteins. Thirdly, binding activity is slightly enhanced by treatment of the 30S subunit with RNase T1 (data not shown), suggesting RNA competition to the binding activity.

As numerous cellular proteins are known to cosediment with the ribosomal fraction under the conventional sedimentation procedures (27), we can not rule out the posibility that the binding protein(s) are not ribosomal protein(s).

The observation that the change in cellular ppGpp level preceds the change in UAS binding capacity and that the binding activity is confind to logarithmically growing cells, may point to dissociate the known growth rate control of rRNA synthesis from cellular ppGpp level per se.

In conclusion, it appears that ribosomes, which have been implicated previously to participate in the control of rRNA gene expression (28), do in fact bind proteins involved in the complex control of the *rrn*A operon. We are currently in the process of isolating the proteins involved in this control. This should shed light on the molecular mechanisms involved in the high transcription level seen with stable RNA genes under favorable growth conditions.

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