Development of a Food-Grade Vector System for Staphylococcus carnosus

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In vivo Synthesis of a Polycistronic Messenger RNA for the Ribosomal Proteins L11, L1, L10 and L7/12 in *Escherichia coli*

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Summary. Sucrose density gradient centrifugation and DNA/RNA hybridization have been used to analyse the mRNA synthesized from the ribosomal protein – RNA polymerase subunits gene cluster *rplKAJL-rpoBC* in *Escherichia coli*. DNA/RNA hybrids obtained from total *E. coli* RNA and specific DNA restriction fragments from this chromosomal area were further subjected to endonuclease S1 digestion. This analysis permits the mapping of the ends of mRNA molecules for specific genes or operons by sizing the S1 resistant hybrids.

Our results show that the predominant mRNA synthesized under conditions of balanced growth from the *rplKAJL-rpoBC* region codes for the four ribosomal proteins L11, L1, L10 and L7/12. This tetracistronic mRNA puts the transcription of the following *rpoBC* genes under the main control of the L11 promoter. Smaller distinct mRNA species could also be detected by this technique. They originate from intercistronic transcription termination and re-initiation as well as from processing of the larger polycistronic mRNA.

Introduction

In *E. coli*, genes for components of the transcriptional and translational machinery are often clustered, forming connected transcription units. Their expression is regulated by complex control mechanisms which operate according to the varying needs for RNA polymerase and ribosomes under different growth conditions (for recent review articles see: Nomura et al. 1977; Maaløe 1979; Matzura 1980). One such gene cluster is located between the *argH* and *purD* locus at positions 89 to 90 min on the *E. coli* chromosome (Bachmann and Low 1980). It contains the genes for ribosomal and transfer RNAs, for the translation elongation factor EF-Tu, for the four ribosomal proteins L11, L1, L10 and L7/12 and for the two RNA polymerase subunits β and β' (Nomura 1976; Lindahl et al. 1977).

The genes for the four ribosomal proteins and the two RNA polymerase subunits in this cluster map adjacent to each other and, therefore, the question arises whether they are arranged in one or more operons, and how their expression is adjusted to each other fulfilling the cell's requirement for their products. Two main transcription units have been proposed to exist in this area: The first begins at the promoter P_{L11} containing the genes *rplK* and *rplA* for the ribosomal proteins L11 and L1, the second begins at the promoter P_{L10} (formerly called "β promoter") including the genes for L10 and L7/12, *rplJ* and *rplL*, as well as for the β and β' subunits, *rpoB* and *rpoC* (Yamamoto and Nomura 1978, 1979; Linn and Scaife 1978). The existence of additional weak promoters between *rplJ* and *rplL* and between *rplL* and *rpoB* has also been reported (Newman et al. 1979; Fiil et al. 1979; Barry et al. 1979). Furthermore, an attenuator signal and an RNase III site have been located between *rplL* and *rpoB*, explaining the five- to sixfold lower rate of mRNA synthesis for the two RNA polymerase subunits than for the ribosomal proteins (Dennis 1977; Barry et al. 1979, 1980; Dennis and Fiil 1979).

In addition to this regulation of transcription, translational control mechanisms have been found to be involved in the regulation of this complex gene cluster. Certain ribosomal proteins are able to repress the synthesis of their own and of other ribosomal proteins which share the same operon in vivo and in vitro by a feedback mechanism that modulates the translation efficiency of the corresponding polycistronic mRNA (Yates et al. 1980; Brot et al. 1980; Dean and Nomura 1980; Fukuda 1980; Nomura et al. 1980).

In most of the experiments elucidating promoter signals, DNA fragments from the *rplKAJL-rpoBC* region were cloned into various vectors and, subsequently, their expression was analyzed under a variety of conditions. The question poses itself, however, to what extent the obtained results can reflect the normal in vivo situation. Post et al. (1979) have already pointed out that the presence of a promoter between *rplA* and *rplJ* (the "β promoter") was demonstrated only under conditions under which the L11 promoter is not functioning, that possibly under normal conditions all the "β operon genes" (*rplJL-rpoBC*) are cotranscribed with the L11 operon genes (*rplKA*) and that the "β promoter" is used only when transcription from an upstream promoter is abolished. If this assumption is correct, one would expect the synthesis of a polycistronic mRNA carrying the information for all six polypeptides and, when attenuation occurs between *rplL* and *rpoB*, of a mRNA for the four ribosomal proteins. Whether such large mRNAs can be detected would mainly depend on the rate at which they are processed to smaller messengers and finally degraded.

In the present work, we have isolated RNA from exponentially growing *E. coli* cells and analyzed by sucrose gradient centrifugation and hybridization to DNA carrying parts of the *rplKAJL-rpoBC* gene cluster. Hybrids obtained with specific DNA fragments which span the region from *rplK* to *rplL* and *rplJ*, respectively, were further subjected to endonuclease S1 treatment. This analysis allows the mapping of the position of the ends of mRNA molecules for specific genes by sizing the hybrids formed with selected restriction fragment probes (Berk and Sharp 1978; Barry

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et al. 1980). Evidence has been obtained that, under conditions of balanced growth, the predominant mRNA synthesized from the *rplKAJL-rpoBC* region carries the information for the four ribosomal proteins L11, L1, L10 and L7/12. Smaller mRNA species can also be detected. Whether they originate from processing of larger molecules or from intercistronic transcription initiation will be discussed.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli K12 W3350 wild-type was grown in M9 medium, supplemented with glucose (0.2%), thiamine (1 μg/ml) and the 20 standard amino acids (each at 60 μg/ml). Strains carrying the plasmids pJC701, pJC703 and pJC720 were obtained from J. Collins.

Preparation of Plasmid DNA and Isolation of Restriction Fragments

Composite ColE1 plasmid DNAs were prepared according to Clewell and Helinski (1969) and Collins et al. (1976). The structure of the plasmids used in this study has been described in detail (Collins et al. 1976; Collins 1979). For the isolation of fragment E (cf. Fig. 1) pJC701 DNA was cut with *Eco*RI. The digested DNA was then subjected to electrophoresis on 6% preparative polyacrylamide gels. Fragments were localized by UV illumination and excised. The gel pieces were placed in dialysis bags and the DNA was recovered by electrophoresis in TE buffer (0.01 M tris·HCl, pH 8, 1 mM EDTA). For the isolation of the *Eco*RI/*Hin*dIII fragment, fragment E was re-cut with *Hin*dIII and the desired fragment was obtained in the same way.

RNA Preparation, Sedimentation and Filter Hybridization

Strain W3350 was grown in supplemented M9 medium and labelled with [^{3}H]uridine (200 μCi/ml, 1.31 μg/ml) for 3 min during exponential growth. Labelling was terminated and RNA was isolated as described earlier (Simon and Matzura 1976). RNA was sedimented through a 4–20% sucrose gradient in NETS buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris.HCl, pH 7.5, 0.2% SDS) (Strohmann et al. 1977). RNA of each fraction was hybridized to denatured plasmid DNA, immobilized on nitrocellulose filters as described (Simon and Matzura 1976). Each hybridization assay contained one filter with 0.2 pmole pJC703 or pJC720 DNA, one filter with ColE1 DNA and one filter without DNA. Following RNase treatment, the radioactivity associated with the filters was determined by liquid scintillation counting. Counts of control filters plus background were subtracted from all other values.

Endonuclease S1 Mapping

These experiments were carried out according to Berk and Sharp (1978) with the following changes: 0.6 μg of fragment E or of the *Eco*RI/*Hin*dIII fragment and 2 to 2.5 mg of total *E. coli* RNA were used per hybridization. RNA was isolated as described without radioactive labelling and hybridized unfractionated in hybridization buffer (0.4 M NaCl, 0.04 M PIPES, pH 6.4, 1 mM EDTA, 80% formamide) to the restriction fragments. The hybridization mixture was incubated at 70° C for 15 min in order to denature the DNA and the DNA/RNA hybrids were formed at 53° C for 4 h. The mixture was then treated with a 90- to 120-fold excess of S1 at 40° C for 1 h. The reaction was stopped by adding the same volume of phenol, equilibrated with 0.1 M Tris·HCl, pH 7.8. After ethanol precipitation, mononucleotides were removed by Sephadex G75 chromatography. S1 resistant material was again ethanol precipitated in the presence of 10 μg tRNA as carrier and separated on neutral 1.5% agarose gels as described by Berk and Sharp (1978).

Chemicals and Enzymes

Chemicals used were analytical grade and obtained from Merck, Darmstadt. Restriction and S1 endonucleases were purchased from Boehringer, Mannheim GmbH.

Results

Hybridization of Fractionated RNA

The first hint of a polycistronic mRNA for L11, L1, L10 and L7/12 was given by DNA/RNA hybridization studies with fractionated *E. coli* RNA and the DNA of the plasmids pJC703 and pJC720 (Collins et al. 1976). Figure 1 shows the map of pJC703 with the inserted *E. coli* DNA which reaches from the *Eco*RI site in *rplK* beyond the end of *rpoC*. pJC720 lacks the E fragment and contains *rpoBC* and only part of *rplL*, missing *rplK*, *rplA* and *rplJ*. When pulse-labelled *E. coli* RNA is hybridized after fractionation through a sucrose gradient to the DNA of one of these plasmids, the hybridization profiles in Fig. 2 result. Two peaks appear in both profiles, one at 21S, the other at 14S. The hybridization to pJC720 is reduced within the two peaks to about 30% as compared to pJC703. Since the two plasmids differ only in their content of genes for the ribosomal proteins, the RNA hybridized in these two peaks must code for these proteins.

In Table 1 we have summarized S values calculated according to a formula given by Kurland (1960) for possible mRNA species from the *rplKAJL-rpoBC* gene cluster. For this calculation we have relied on two DNA sequence analyses; the first (Post et al. 1979) spans the region from about 180 base pairs (bp) before L11 to the beginning of the coding sequence for the β subunit, the second (Ovchinnikov et al. 1980) covers the β and a small part of the β′ genes. A comparison of the calculated S values with the values of the two sedimentation peaks suggests that the 21S RNA could code for all the four ribosomal proteins whereas the 14S peak could contain mRNA for L11 and L1 and/or L10 and L7/12.

Endonuclease S1 Mapping

In order to determine the nature of the two mRNA species hybridized to the two plasmids, total RNA, isolated from exponentially growing *E. coli* cells, was hybridized to specific DNA fragments from the *rplKAJL* region. The hybrids were then subjected to endonuclease S1 digestion which degrades single-stranded DNA or RNA but leaves double-stranded nucleic acids intact (Vogt 1973).

The first such experiment was performed with the DNA of fragment E which runs from the *Eco*RI site within the first half of *rplK* to the *Eco*RI site in about the middle of *rplL* including *rplA* and *rplJ* (Fig. 1). This fragment is 2.164 bp long. It appears in Fig. 3A, Track 1 as the third band of a pJC701/*Eco*RI digest. If there exists a polycistronic mRNA for the four ribosomal proteins, the whole fragment should be protected and,

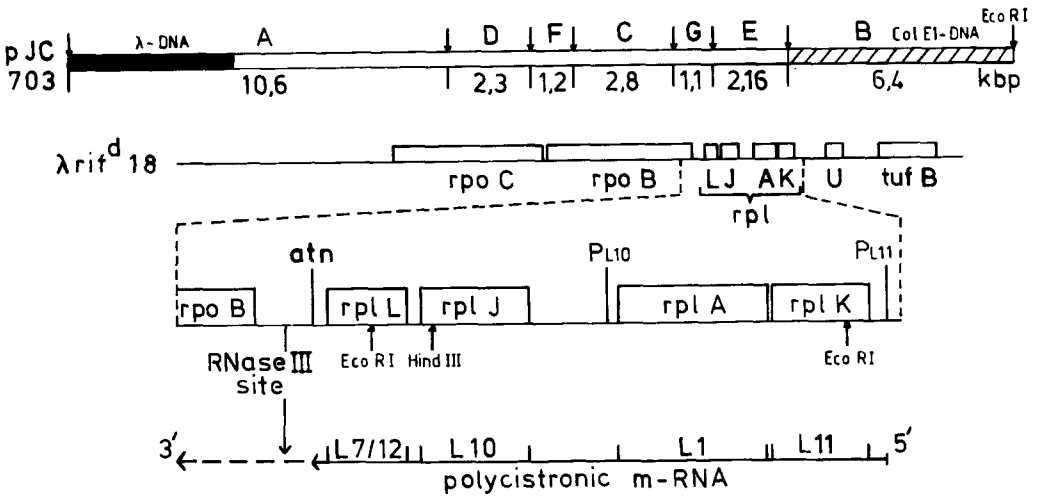

Fig. 1. Genetic and Physical Map of the *E. coli* DNA Carried by the Composite ColE1 Plasmid pJC703 and of Part of the Corresponding DNA in *λrif*d18. Indicated are restriction sites used for the present work. The approximate lengths of the ribosomal protein genes and the positions of the promoter and attenuator signals as well as the RNaseIII site are given according to Post et al. (1979) and Barry et al. (1980). pJC720 lacks fragment E and pJC701 fragments D and A

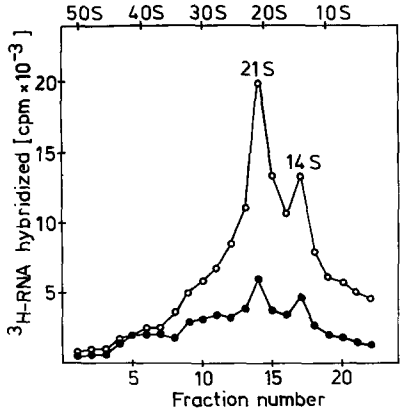

Fig. 2. Hybridization of fractionated *E. coli* RNA to pJC703 and pJC720 DNA. The two profiles were obtained by combining the results of four independent hybridization experiments, as described in Materials and Methods, with each plasmid, and counts hybridized were normalised to the same input. ○ Hybridization to pJC703; ● Hybridization to pJC720

after S1 digestion, the hybrid should have the same size as the fragment. In Track 4 of the same figure the resulting DNA/RNA hybrids are shown. Several bands can be recognized there, however, the predominant one runs at a position corresponding to fragment E in Track 1. The determination of the length of this hybrid by means of several standards (Fig. 3A, Track 5) yields approximately 2,110 bp. We cannot explain the seeming difference of about 50 bp, but it might be that, due to an altered tertiary structure, a DNA/RNA hybrid runs slightly faster in an agarose gel than double stranded DNA of the same size. From the length of this hybrid it is obvious, however, that the mRNA in it has been tetracistronic with the information for the four ribosomal proteins L11, L1, L10 and L7/12, initiated at the L11 promoter. It could thus correspond to the RNA in the 21S peak of Fig. 2. Further evidence for the existence of this polycistronic mRNA is provided by hybridization of total *E. coli* RNA to the *Eco*RI/*Hin*dIII fragment which can be obtained from fragment E by an additional *Hin*dIII digest. This fragment has a length of 1,876 bp and lacks *rplL* and part of *rplJ* (Fig. 1). The corresponding hybrid would, therefore, be

Table 1. Lengths, molecular weights and sedimentation coefficients for various polycistronic mRNA species

mRNA	Length (nucleotides)	Molecular weight (kdaltons)	S values
L11, L1	1,251	41	13.6
L10, L7/L12	1,368	44	14.3
L11, L1, L10, L7/12	2,637	86	20.6
β and β'	8,890	289	40.7

The lengths of the RNA molecules were calculated on the basis of nucleotide sequences by Post et al. (1979) and Ovchinnikov et al. (1980). For the start signals of possible mRNA positions proposed by Post et al. (1979) were taken. The L11/L1-mRNA was assumed to terminate at a position around 1,330 (numbering according to Post et al. (1979)) before the Pribnow box of P_{L10}. The stop signal for both the L10/L7/12- and the tetracistronic mRNAs was taken at the attenuator position 2,726 (Barry et al. 1980). For the $\beta\beta'$-mRNA we relied on the nucleotide sequence by Ovchinnikov et al. (1980), beginning at the RNaseIII site before *rpoB* (Barry et al. 1980) and terminating at the start of the coding sequence for the β' gene. For the rest, the β' polypeptide (molecular weight 165,000) was converted to the corresponding RNA, assuming an average molecular weight of 110 per amino acid. The molecular weights of the RNAs were then deduced taking an average molecular weight of 325 per nucleotide, and the S values were calculated according to Kurland (1960)

expected to migrate at a position of approximately 1,800 bp. In Fig. 3B, Track 2 we can see a strong band at a position of 1,820 bp besides several smaller bands. Thus, by hybridization to the smaller *Eco*RI/*Hin*dIII fragment and subsequent S1 digestion, the 3'-end of the tetracistronic mRNA is degraded and a hybrid, 288 bp smaller than the first one, results.

There are several weaker bands visible in both tracks (Fig. 3A, Track 4 and 3B, Track 2). For the comparison of these shorter hybrids Track 4 of Fig. 3A and Track 2 of Fig. 3B were scanned (see Fig. 3C and D). Three of the hybrids have the same size, namely about 1,290, 1,180 and 1,020 bp, and two have different lengths, in Fig. 3A, 4 and 3C of about 900 and 800 bp, in Fig. 3B,2 and 3D of about 640 and 540 bp. These smaller RNA species could give rise to the 14S peak in Fig. 2.

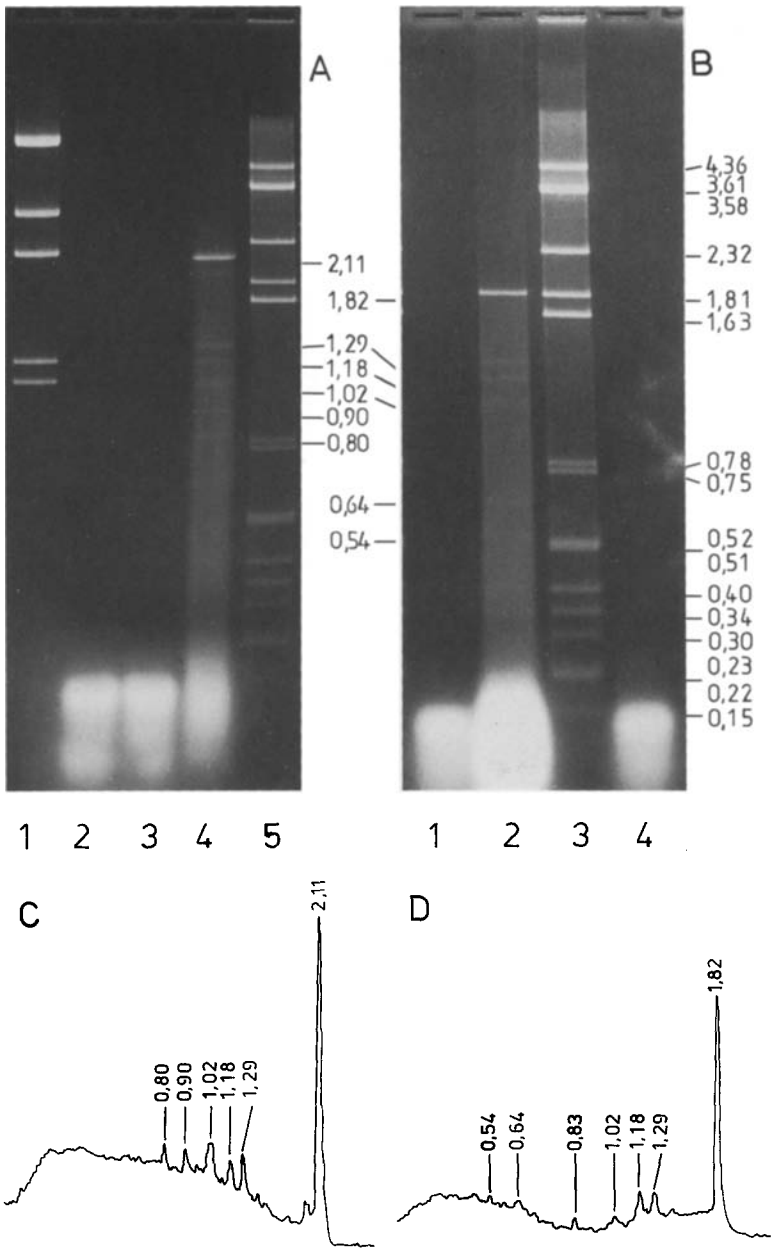

Fig. 3A–d. Agarose Gel Electrophoresis of DNA Restriction Fragments and DNA/RNA Hybrids after S1 Digestion. **A** S1 mapping with fragment E. Track 1: *Eco*RI digest of plasmid pJC701; track 2: fragment E, treated as the hybridization mixture; track 3: *E. coli* RNA, treated as the hybridization mixture; track 4: hybridization mixture of fragment E and total *E. coli* RNA; track 5: molecular weight standards obtained from plasmid pBR322 by various restriction degradations: *Hin*dIII (4.36 kbp), *Bgl*I (2.32, 1.81, 0.23 kbp), *Hinf* (1.63, 0.52, 0.51, 0.40, 0.34, 0.30, 0.22, 0.15, 0.075 kbp), *Eco*R1/*Pst*I (3.61, 0.75 kbp), *Hin*dIII/*Pst*I (3.58, 0.78 kbp). **B** S1 mapping with the *Hin*dIII/*Eco*RI fragment. Track I: *Hin*dIII/*Eco*RI fragment, treated as the hybridization mixture; track 2: hybridization mixture of the *Hin*dIII/*Eco*RI fragment and total *E. coli* RNA; track 3: molecular weight standards as track 5 in A; track 4: *E. coli* RNA, treated as the hybridization mixture. The lengths of the fragments and hybrids are given in kilo base pairs (kbp). **C** Densitometer scan of Track 4 from A. **D** Densitometer scan of Track 2 from B

Hybrids which were generated by mRNAs covering the end of fragment E within *rplK* should become about 290 bp shorter in the *Eco*RI/*Hin*dIII hybridization assay. Therefore, it is possible to differentiate between RNA species which carry promoter proximal or promoter distal sequences by comparing the size of the hybrids in the two experiments. The difference between the 900 bp and the 640 bp hybrid and between the 800 bp and the 540 bp hybrid is in both cases 260 bp, thus corresponding to the different lengths of the two fragments used as hybridization probes. These four hybrids, therefore, contain RNA from promoter distal sequences. After S1 digestion, the 3′-ends of these RNAs are located at the *Eco*RI site in *rplL* and the *Hin*dIII site in *rplJ*, respectively. Since the two larger hybrids of about 1,290 and 1,180 bp appear in both S1 experiments, they can be assumed to originate from promoter proximal sequences. The sums of the 1,290 and 800 bp hybrids as well as of the 1,180 and 900 bp hybrids yield approximately the same value, and this corresponds to the large hybrid of about 2,110 bp. We can, therefore, assume that these four minor hybrids contain processing products of the tetracistronic mRNA. By counting from the 3′-ends of the hybrids the two possible processing sites lie within the intercistronic region between *rplA* and *rplJ*. Whether such processing is a real in vivo event or occurs during the isolation of the RNA cannot be distinguished at present.

There is a fifth hybrid band present in both tracks at a position of about 1,020 bp which appears to be a double band in the experiment with fragment E (Fig. 3A,4 and 3C). Comparing the length of this RNA with known transcription signals of the DNA sequence it could have two origins: First, it may be initiated at P_{L11} and terminated before P_{L10}; second, it may be initiated at P_{L10} and terminated somewhere behind the *Eco*RI site in *rplL*, presumably at the attenuator before *rpoB*. In the second case this hybrid would be expected to disappear in the second hybridization assay, giving rise to a new band around 800 bp.

Careful comparison of the densitometer scans of the two

gel tracks shows that the 1.020 bp hybrid band in Fig. 3A,4 and 3D is slightly broader and more pronounced than in Figs. 3B,2 and 3D, and a sixth band appears in Fig. 3D at a position of about 830 bp. The double band at 1,020 bp may, therefore, contain two RNA species, one being the product of a real termination after *rplA*, the other of re-initiation at P_{L10}. Both RNAs would also appear in the 14S peak of Fig. 2.

Discussion

In the present communication evidence is presented for the existence of a polycistronic mRNA carrying the information for the four ribosomal proteins L11, L1, L10 and L7/12. This mRNA appears to be by far the major transcript from the *rplKAJL* gene cluster and puts the transcription of the following *rpoBC* genes under the main control of the L11 promoter. Thus, the assumption made by Post et al. (1979) that all the "β operon" genes might be co-transcribed with the L11 operon genes has been confirmed. To avoid confusion, we propose not to use the term "β promoter" anymore but instead P_{L11} or P_{L10} for the corresponding transcription initiation site. The existence of a second promoter in this gene cluster, namely P_{L10}, has not been ruled out but could be confirmed, too, although P_{L10} does not seem to contribute significantly to transcription initiation when P_{L11} is functioning. According to the DNA sequence there are only about 30 bp between the end of the coding sequence for L1 and the Pribnow box of P_{L10}, and there is no sequence common to known termination signals, unless the sequence of dyad symmetry around P_{L10} functions as such (Post et al. 1979). Therefore, we can assume that RNA polymerase does not usually terminate after *rplA* but continues transcription over P_{L10} giving rise to the observed tetracistronic mRNA, and that transcription termination and re-initiation occur only occasionally in this area under equilibrium conditions. The possibility that P_{L10} is a special promoter of the λrif^d18 phage which was the source for construction of the used plasmids can be ruled out by the findings that λrif^d18 accurately represents the corresponding region of the *E. coli* K12 chromosome (Kirschbaum and Konrad 1973; Collins et al. 1976; Newman and Hayward 1980).

Farther downstream in the DNA sequence around position 1,600 is another region with dyad symmetry which could provide a recognition site for some hypothetical processing enzyme. It could account for the appearance of the two hybrid bands at about 1,290 and 800 bp in Fig. 3A,4 or 540 bp in Fig. 3B, 2, respectively. For the hybrid bands at 1,180 and 900 bp or 640 bp, respectively, we have to postulate another RNase-sensitive site shortly before position 1,500 in Post et al. (1979) nucleotide sequence, and a hairpin-loop-like structure can be constructed in this area.

One interesting feature in the regulation of this complex operon is the recently detected translational control. Relevant for our work are the findings that the ribosomal protein L1 is able to inhibit translation of the L11/L1-message and that L10 or a complex of L10 and L7/12 controls the synthesis of these two proteins (Brot et al. 1980; Yates et al. 1980; Dean and Nomura 1980; Fukuda 1980). Nomura et al. (1980) have developed a model according to which the repressor ribosomal protein binds to parts of the mRNA including its beginning thus hindering its further translation. The finding that L1 does not inhibit the synthesis of L10 or L7/12 only shows that there is independent translation initiation for those two proteins. In contrast to the assumption of Yates et al. (1980), however, we have shown that here also the unit of transcription is different from the units of autogenous translational regulation.

Does there exist a polycistronic mRNA for the four ribosomal proteins and the two polymerase subunits β and β'? Barry et al. (1980) have shown that RNA polymerase is able to transcribe through the intercistronic region between *rplL* and *rpoB* into *rpoB*. Because of the RNaseIII site before *rpoB* it would have been very unlikely that we could have detected such large mRNA in our experiments. Only the shoulder around 40S in the sucrose gradient profiles (Fig. 2) hints at a $\beta\beta'$-mRNA which could be the processing product of the larger polycistronic mRNA.

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