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T7 RNA polymerase directed expression of the *Escherichia coli* *rrn*B operon

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Plasmids having *Escherichia coli* ribosomal DNA sequences under control of a promoter for T7 RNA polymerase have been constructed. Transcription of the rDNA sequences is dependent on T7 RNA polymerase because the tandem promoters for *E. coli* RNA polymerase, normally used to direct transcription of these sequences, have been removed. The entire 16S, 23S and 5S coding sequences from the *rrn*B operon can be efficiently transcribed by T7 RNA polymerase *in vitro* to yield full-length 30S precursor RNA. When such plasmids are placed into an *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase under control of the *lac* UV5 promoter, high-level synthesis of rRNAs from the plasmid can be induced by adding IPTG to exponentially growing cells. Subsequent addition of rifampicin to inhibit further initiation of transcription by *E. coli* RNA polymerase provides a simple method to study the fate of plasmid-coded rRNAs in the complete absence of host-coded rRNA synthesis. Gel electrophoretic analysis demonstrated that the rRNAs synthesized by T7 RNA polymerase in the presence of rifampicin are processed to their mature forms and assembled into ribosomal particles for at least 35 min after rifampicin addition. T7 RNA polymerase is also capable of efficient transcription of the entire *rrn*B operon in the reverse direction.

Key words: plasmids/ribosomes/rRNA/transcription/T7 RNA polymerase

Introduction

The primary and secondary structures of *Escherichia coli* rRNAs have been known for several years (Brimacombe, 1981; Noller and Woese, 1981). The cloning of two of the seven operons which code for 16S, 23S and 5S rRNAs has made possible detailed studies into the mechanisms which regulate rRNA synthesis and helped delineate control determinants with increasing precision (Brosius *et al.*, 1981; Morgan, 1982; Sarmientos and Cashel, 1983). Much less is known about the function of specific regions of the rRNAs. Attempts to study rRNA function by introducing mutations into cloned rRNA genes have demonstrated the constitutive expression of mutant rRNAs leads to an increase in host generation time and, as a result, mutant strains are easily overgrown by cells that either have lost the plasmid or contain plasmids with spontaneous alterations relieving growth inhibition (Gourse *et al.*, 1982). A conditional rRNA gene expression system utilizing *E. coli* RNA polymerase has recently been described which allows regulated expression of plasmid-encoded rRNAs (Gourse *et al.*, 1985). Presumably this system will permit the isolation of mutations in rRNAs in a more stable background. However, expression of plasmid-encoded rRNAs independent of transcription of rRNA genes on the chromosome would still require the use of a modified maxicell system (Stark *et al.*, 1982).

A potential way to circumvent many of the problems inherent in using genetic approaches to study rRNA function might be to eliminate entirely the need to use *E. coli* RNA polymerase for expressing plasmid-encoded rRNA genes. The approach we have used here was to replace the *E. coli* promoters normally used for transcription of the *rrn*B operon, coding for 16S, 23S and 5S rRNAs, with a promoter for T7 RNA polymerase. Transcription of the operon should now be dependent on T7 RNA polymerase since *E. coli* RNA polymerase does not utilize T7 promoters. T7 RNA polymerase does not seem to be subject to the factors that cause *E. coli* RNA polymerase to terminate transcription (Holmes *et al.*, 1983; J.J.Dunn and F.W.Studier, unpublished data) and we therefore expected that the enzymes would be capable of producing complete transcripts of the entire *rrn*B operon.

To direct expression *in vivo*, the recombinant plasmids were moved into a host strain having a chromosomal copy of the gene for T7 RNA polymerase under control of the *lac* UV5 promoter (Studier and Moffatt, 1986). Isopropyl β-D-thiogalactopyranoside (IPTG) was used to derepress the *lac* UV5 promoter and allow active T7 RNA polymerase to accumulate in the cells. Since T7 RNA polymerase, unlike *E. coli* RNA polymerase, is rifampicin resistant (Chamberlin *et al.*, 1970), we could selectively follow the fate of rRNAs synthesized by T7 RNA polymerase by using rifampicin to inhibit chromosomal rRNA synthesis by *E. coli* RNA polymerase. Our results show that the T7 system will be useful for studying expression of plasmid-coded rRNAs in the complete absence of host-coded rRNA synthesis.

Results

Construction and in vitro transcription of recombinant plasmids

Plasmid pKK3535 (Figure 1) contains the entire *rrn*B operon of *E. coli*, coding for 16S, 23S and 5S rRNAs plus a $tRNA_{Glu2}$ spacer sequence, on a 7508-bp *Bam*HI fragment derived from the transducing phage λ*rif*[d]18 (Brosius *et al.*, 1981). The primary sequence of the entire fragment is known (Brosius *et al.*, 1981) and we will refer to nucleotide positions of cloned rDNAs according to their location within the 7.5-kb *Bam*HI fragment, not according to their position in individual plasmids. Immediately preceding position 3247 near the 5′ end of the tRNA spacer is a unique recognition site for *Xba*I. Cutting pKK3535 with *Xba*I and *Bam*HI released a fragment coding for 23S and 5S rRNAs that could be cloned directly behind the T7 promoter in plasmid pAR2156 in the correct orientation. When the resulting plasmid, pAR3084, was linearized with *Bam*HI and transcribed by purified T7 RNA polymerase, two high mol. wt transcripts were produced (Figure 2). The larger has the mobility expected for a full-length transcript to the end of the linear DNA, ~4288 bases, while the smaller is of a size consistent with T7 RNA polymerase terminating transcription when it reaches the transcriptional ter-

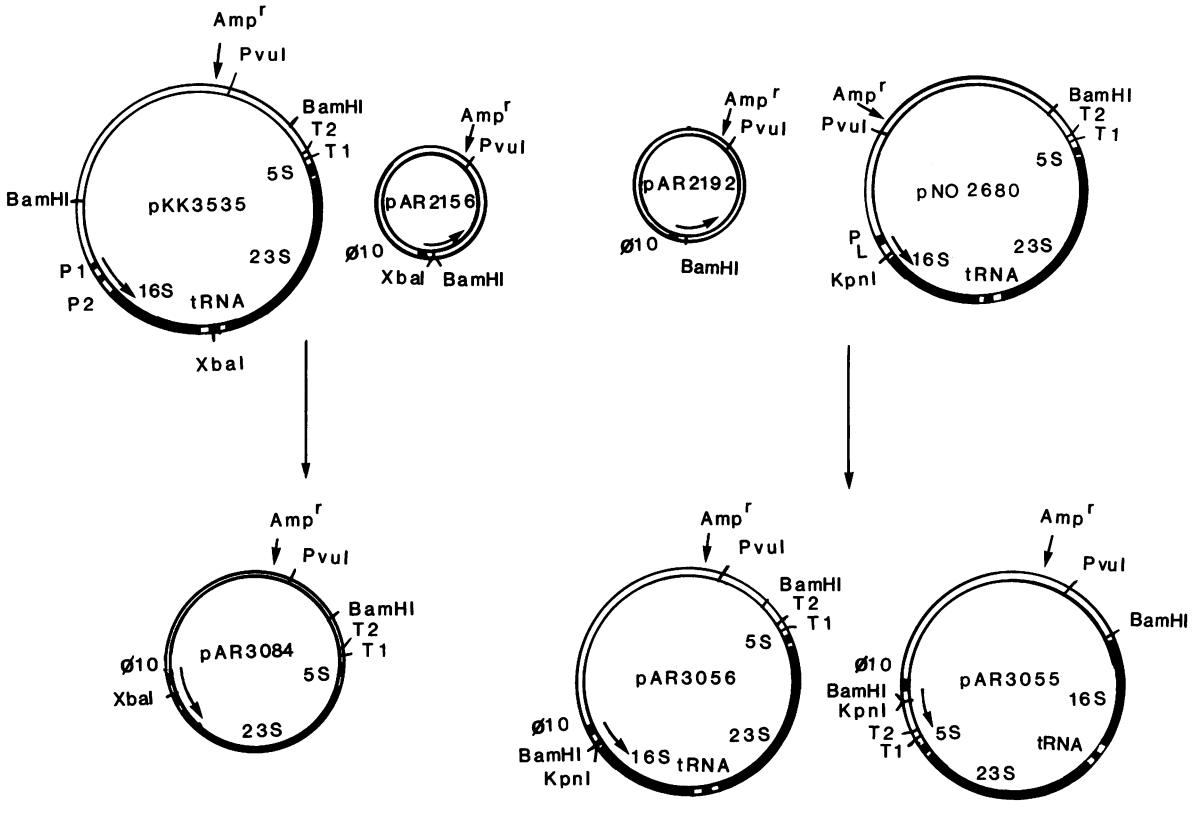

Fig. 1. Diagram of the construction of pAR3055, 3056 and 3084. The various steps and relevant restriction enzyme sites used in the construction of the T7 promoter/rDNA plasmids are shown. $\phi 10$ represents the promoter for T7 RNA polymerase. pAR2156 contains nucleotides 22 880–22 998 of T7 DNA while pAR2192 contains nucleotides 22 880–22 928 (Dunn and Studier, 1983). DNA sequence analysis (Maxam and Gilbert, 1979) of the recombinant plasmids predicts that the transcripts begin with the following sequences: pAR3056 pppGGGAGACCACAACGUUUCCCUCUAGCGGGAUCCGGUACCGG$_{1369}$, and pAR3084 pppGGGAGACCACAACGGUUUCCCU$_{3241}$. G$_{1369}$ and U$_{3241}$ are the first residues from the rDNA in the respective transcripts.

mination signal (T1) adjacent to the 3′ terminus of the 5S RNA gene, ~3440 bases. Although the T1 terminator and the one terminator for T7 RNA polymerase in T7 DNA (Dunn and Studier, 1983) share little sequence homology, both have in common a potential for forming large hairpin structures (20 and 15 bp, respectively) that end in a stretch of T residues. However, neither terminator is completely efficient.

Plasmid pNO2680 was used as the source of DNA to clone the entire structural portion of the *rrn*B operon under control of a T7 promoter. This plasmid was cut at its unique *Kpn*I site ahead of the 16S rRNA gene, ligated to a synthetic *Bam*HI–*Kpn*I linker–adaptor and then cut with *Bam*HI. The fragment containing the structural portion of the operon, but lacking the natural *E. coli* promoters, was purified and cloned into a T7 promoter vector, pAR2192, which has a single *Bam*HI site downstream of the transcriptional start site. This plasmid lacks the *tet* promoter for *E. coli* RNA polymerase which could otherwise direct opposing transcription. Full-length inserts were obtained in both possible orientations (Figure 1): transcription from the T7 promoter in pAR3056 produces rRNA of the correct polarity, while from pAR3055 only anti-sense rRNA would be produced. DNA sequence analysis (Maxam and Gilbert, 1979) of the promoter/rDNA junction in pAR3056 revealed that the rDNA sequence begins 41 bp past the start point for T7 transcription at nucleotide position 1369, and that the unique recognition site for *Kpn*I was retained during construction (see Figure 1).

As can be seen in Figure 2, each DNA is efficiently transcribed by T7 RNA polymerase to produce full-length transcripts of the entire *rrn*B operon. As was expected from our previous results with pAR3084, most pAR3056 transcripts terminate at T1 although some molecules extend past T1 to the *Pvu*I site used to linearize this set of templates. Only weak termination was observed at the anti-sense T1 region in pAR3055, even though in this polarity the RNA still has the potential for forming a 20 bp long duplex with a single A/C mismatch. However, in this orientation the potential hairpin is not immediately followed by a stretch of T residues.

RNase III processing of in vitro transcripts

In *E. coli* the first step in processing of primary transcripts from ribosomal operons is cutting of the nascent RNA by RNase III (Dunn and Studier, 1972; Nikolaev *et al.*, 1973). As shown in Figure 2, the rRNAs synthesized by purified T7 RNA polymerase from pAR3056 and pAR3084 are also cleaved by RNase III to generate RNAs of sizes consistent with cleavage at the known 16S and 23S RNase III processing sites in the *rrn*B operon (Brosius *et al.*, 1981; Ginsburg and Steitz, 1975; Lund and Dahlberg, 1979; Bram *et al.*, 1980). In addition, RNase III apparently cleaves at the T1 stem–loop structure when it is present in the full-length run off RNAs. Apirion and co-workers (Szeberenyi and Apirion, 1984) also have observed RNase III cleavage in the *rrn*B termination stem. The only specific RNase III site detected in the pAR3055 anti-sense rRNA transcript also seems to be at the T1 region (data not shown) which, even when transcribed in this orientation, still has the potential for forming a substantial duplex structure.

T7-directed rRNA synthesis in vivo

To investigate synthesis of rRNAs by T7 RNA polymerase *in vivo*, we placed pAR3056 in *E. coli* BL21(DE3), a rifampicin-sensitive strain having a chromosomal copy of the gene for T7

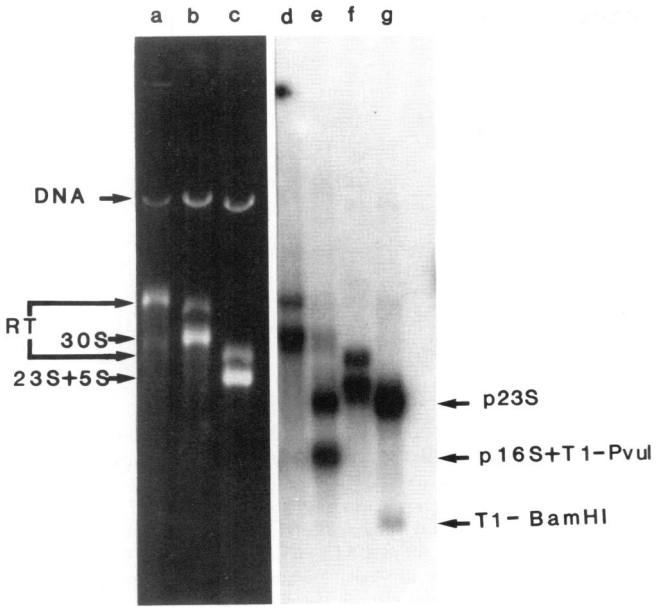

Fig. 2. Synthesis and RNase III processing of rRNAs *in vitro*. Reaction mixtures containing template DNAs linearized with *Pvu*I were incubated as described in Materials and methods. **Lanes a–c** show RNAs present in a 2.5 μl aliquot after 30 min of incubation at 37°C as detected by fluorescence using pAR3055, 3056 and 3084 as template. **Lanes d–g** show an autoradiogram of the RNase III digestion products of the pAR3056 (**e**) and pAR3084 (**g**) transcripts plus their respective no RNase III controls (**d** and **f**). Identification of RNAs indicated on the sides of the patterns is based on mobility relative to RNAs synthesized by T7 RNA polymerase from T7 DNA which were electrophoresed on the same gels (not shown). Bands indicated on the left side of the patterns are: DNA, template DNA; RT, read-through from promoter to end of linear DNA; 30S, transcript from φ10 to T1, containing 16S, 23S and 5S rRNA sequences; 25S+5S, transcript ending at T1 containing 23S and 5S rRNA sequences. Bands indicated on the right side of the patterns are those derived from RNase III cleavage: p23S, p16S and RNAs that have their 5′ ends generated by RNase III cleavage near T1 ending at either the *Pvu*I or *Bam*HI sites used to linearize the DNAs. T1-*Pvu*I is ~1400 bases long, T1-*Bam*HI should be ~850 bases long.

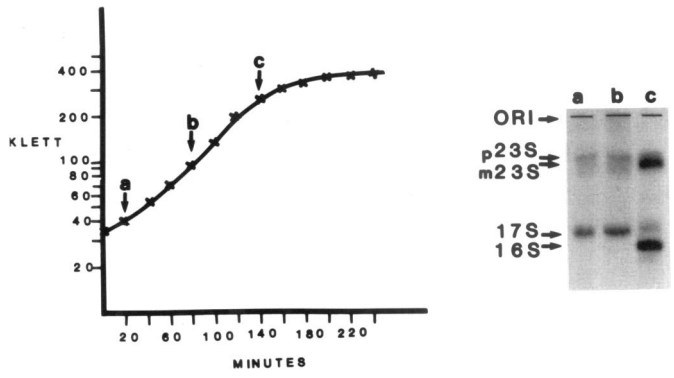

Fig. 3. Synthesis of rRNAs by T7 RNA polymerase *in vivo*: effect of growth conditions on processing of rRNA. BL21(DE3)/pAR3056 cells were induced with IPTG for 45 min at early, mid and late log phases of growth (points, a, b and c of the growth curve, respectively) as described in the text. 5 min after addition of rifampicin (150 μg/ml) the cells were labeled for 20 min with $^{32}PO_4$ and the RNAs separated by electrophoresis on composite gels using Peacock's Tris-borate EDTA buffer system (Peacock and Dingman, 1968). **Lanes a–c** in the autoradiogram at the right show rRNAs of cells induced at points a, b and c in the growth curve.

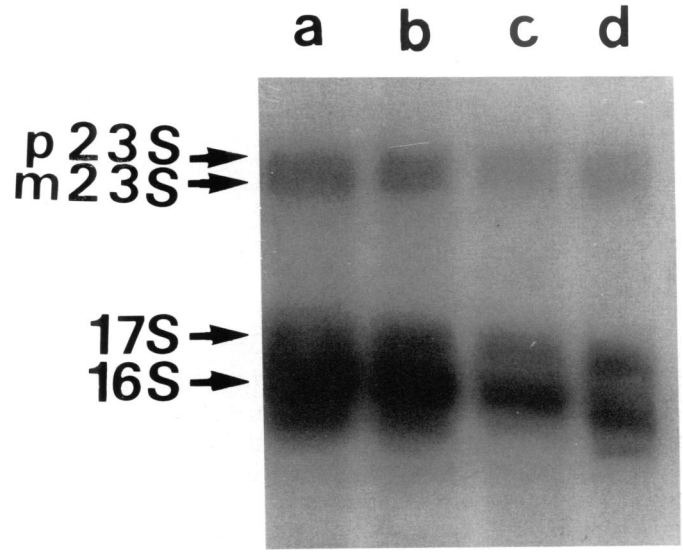

Fig. 4. Synthesis of rRNAs by T7 RNA polymerase *in vivo:* effect of rifampicin incubation time on processing of rRNA. BL21(DE3) pAR3056 cells were induced with IPTG as described in Materials and methods. At various times after addition of rifampicin, individual samples were labeled with $^{32}PO_4$ and the RNAs separated by electrophoresis as in Figure 3. **Lanes a–d** show RNAs labeled at 5, 10, 20 and 35 min, respectively, after addition of rifampicin.

RNA polymerase under control of the *lac* UV5 promoter. A culture of BL21(DE3) harboring pAR3056 was grown overnight in ZPM medium containing 10% L broth. The culture was diluted one part to two parts with fresh ZPM, 10% L broth, 0.2% casamino acids and 0.2% glucose. The cells began to grow immediately (see Figure3, growth plot). At the times indicated in Figure 3 (a, b and c) 10 ml aliquots of the culture were transferred to new flasks and T7 RNA polymerase synthesis induced by addition of 0.5 mM IPTG. Aliquots a, b and c represented cells in early, mid and late log phases of growth, respectively. After 45 min the cultures received rifampicin (150 μg/ml) to inhibit the *E. coli* RNA polymerase. After 5 min with rifampicin the plasmid-coded rRNA synthesis was followed by labeling the cultures with $^{32}PO_4$ for 20 min and analyzing the distribution of labeled RNAs by gel electrophoresis. As can be seen in the resulting autoradiogram (Figure 3), the plasmid-coded *rrn*B operon was transcribed in all three samples but the 16S and 23S rRNAs were processed to their mature forms only in sample c, where IPTG was added to the culture in the late-log phase of growth.

In a subsequent experiment we extended the length of rifampicin treatment prior to labeling plasmid-coded rRNAs with $^{32}PO_4$ while other conditions remained as defined by sample c. As can be seen in Figure 4, the 16S and 23S rRNAs continue to be labeled and processed to their mature forms for at least 35 min after addition of rifampicin. 5S rRNA and a tRNA species that is presumably $tRNA_{Glu2}$ also continue to be synthesized and processed (data not shown). To confirm that T7 RNA polymerase was responsible for the continued synthesis of rRNAs in the presence of rifampicin, a similar experiment was performed using plasmid pAR3056-*Sma*I which contains a 770-bp deletion in the 16S rDNA. This deletion increases the electrophoretic mobility of the transcript from the 16S region of the plasmid (Gourse *et al.*, 1982), thereby allowing us to distinguish between host- and plasmid-coded 16S transcripts. As can be seen in Figure 5, only the faster migrating, plasmid-coded form of 16S rRNA continues to be labeled after rifampicin was added. Little, if any, of the faster migrating form of 16S rRNA could be detected when uninduced cells were labeled (data not shown), even though some

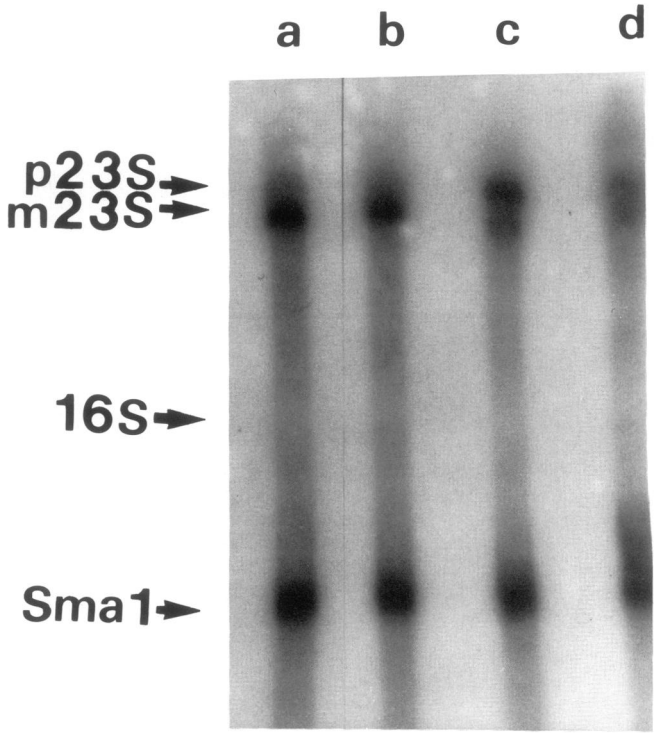

Fig. 5. Synthesis of rRNA in BL21(DE3) carrying plasmid pAR3056-*Sma*I. Cells harboring pAR3056-*Sma*I, having a 770-bp deletion in the 16S rRNA, were grown and labeled as described in Figure 3. Times of labeling and gel electrophoretic conditions are the same as in Figure 3.

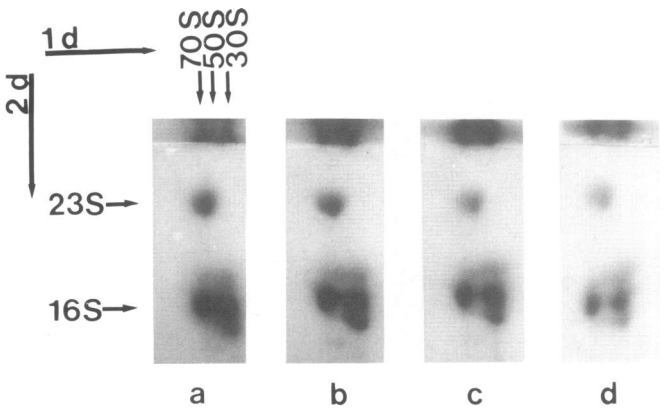

Fig. 6. Two-dimensional electrophoretic analysis of rRNA particles produced in induced BL21(DE3)/pAR3056 cells. Cells were labeled as described in Materials and methods and then lysates prepared under conditions that preserve ribosome integrity (Stark *et al.*, 1982; Dahlberg, 1982). Samples were initially subjected to electrophoresis in a composite gel containing 10 mM $MgCl_2$, 30 mM KCl and 25 mM Tris-HCl, pH 7.8, to separate 70S ribosomes and ribosomal subunits. The first dimension of electrophoresis is from left to right in the autoradiogram shown at the top of this figure. The RNAs were re-electrophoresed in a second dimension (top to bottom) in Peacock's buffer (Peacock and Dingman, 1968) after deproteinization with SDS. The various bands of ribosomes and free rRNAs are indicated. Samples in gels **a**–**d** were labeled at 5, 10, 20 and 35 min after addition of rifampicin, as in Figure 4. Note the reduced amounts of 23S rRNA in 70S particles in gels **c** and **d**, consistent with the reduced levels of m23S rRNA in lanes c and d of Figure 4.

T7 RNA polymerase is known to be produced in the absence of inducer (Studier and Moffatt, 1986). Apparently the background level of rRNA synthesis by T7 RNA polymerase is too low to be detected by this type of analysis.

Processing and assembly of T7/rRNA transcripts in vivo

From the RNA patterns shown in Figures 3 and 4, it is apparent that substantial portions of the RNAs synthesized from pAR3056 are being processed to their mature forms during the 20 min labeling period. This observation implies that most, if not all, of the processing enzymes and ribosomal proteins continue to be available for a period of time after rifampicin addition, since maturation of precursors 16S, 23S and 5S rRNAs occurs only in ribonucleoprotein particles (Schlessinger, 1980). To determine directly whether the RNAs transcribed by T7 RNA polymerase were being assembled into ribosomal particles, IPTG-induced cells were labeled for 20 min with $^{32}PO_4$ beginning at 5, 10, 20 and 35 min after rifampicin was added, and the lysates were electrophoresed under conditions which permit separation of 70S ribosomes and the 50S and 30S subunits (Stark *et al.*, 1982; Dahlberg, 1982). After soaking in buffer containing SDS to release the RNAs, individual gel strips were subjected to electrophoresis in a second dimension to resolve the 16S and 23S rRNAs. The resulting autoradiograms (Figure 6) demonstrate that 16S and 23S rRNAs not only continue to be labeled after addition of rifampicin, but also that they are assembled into particles with mobilities characteristic of 70S ribosomes and subunits. Furthermore, virtually no label was observed in the regions of the first-dimension gels where protein-free rRNAs are known to migrate (Stark *et al.*, 1982); suggesting either that assembly of the pulse-labeled rRNAs into ribosomal particles is rather efficient or that any unassembled rRNA is rapidly degraded.

Discussion

We have placed a ribosomal operon under control of a T7 promoter in a plasmid, and used T7 RNA polymerase to direct high level transcription of the cloned rRNA genes *in vitro* and *in vivo*. Transcription *in vitro* is very efficient and large amounts, up to 1 mg/ml, of ribosomal precursor RNAs or anti-sense transcripts can be synthesized routinely. Inside the cell, high-level transcription of the *rrn*B operon linked to a T7 promoter is obtained simply by adding IPTG to growing cells to induce synthesis of active T7 RNA polymerase from a chromosomal copy of its gene under control of the *lac* UV5 promoter. Indeed, T7-directed transcription of pAR3056-*Sma*I rapidly produces, in <30 min, the faster migrating form of 16S rRNA in amounts sufficient for detection in stained gels and its rate of synthesis eventually becomes approximately equivalent to that of normal 16S rRNA synthesis (data not shown). Once sufficient amounts of T7 RNA polymerase accumulate in the cell, the host RNA polymerase can be inactivated with rifampicin, thereby eliminating further transcription of host-coded rDNAs and allowing analysis of T7-directed rRNA synthesis independent of host rRNAs. Our results show that T7 RNA polymerase continues to transcribe the T7 promoter-linked copy of the *rrn*B operon for at least 35 min after addition of rifampicin and that during this period the transcripts continue to be processed and assembled into ribosomes. Processing and assembly require several different enzymes and >50 different ribosomal proteins. These conditions appear to be met only when IPTG is added to cells in the late-log phase of growth. We have no explanation for this fortunate but peculiar requirement.

Because there are no large pools of free ribosomal proteins in *E. coli* (Fallon *et al.*, 1979), addition of rifampicin might have been expected to stop the assembly of rRNAs synthesized by T7 RNA polymerase into ribosomal particles. Work from Nomura's laboratory suggests an explanation for the continued assembly

of ribosomal particles in the presence of rifampicin; continued synthesis of rRNAs by T7 RNA polymerase may result in a remarkably prolonged translational half-life of mRNAs coding for ribosomal proteins by preventing feedback regulation (Nomura *et al.*, 1984). Preliminary experiments indicate that incorporation of [^{35}S]methionine in the presence of rifampicin in BL21(DE3) cells harboring the pAR3056 plasmid is prolonged if the cells are first induced with IPTG, a result consistent with *rrn*B transcription affecting stability of ribosomal proteins.

The cloning of an operon for 16S, 23S and 5S rRNAs under control of a T7 promoter, coupled with the ability specifically to target expression of the cloned genes *in vitro* and *in vivo*, should make mutational analysis of rRNA function much easier. Although we have concentrated our present studies on demonstrating that T7 RNA polymerase can transcribe the entire *rrn*B operon, it also should be possible to construct clones that express only limited portions of the operon in either polarity. Such clones might have advantages for studying protein–rRNA interactions and regulation of rRNA and protein synthesis.

Materials and methods

Bacteria and plasmids

E. coli HMS174 ($r_{K12}^-m_{K12}^+$*rec*A1 *rif*^{R}) (Campbell *et al.*, 1978) was used as the initial host for plasmid strains, which were all derived from pBR322 by inserting fragments of T7 DNA containing the ϕ10 promoter of T7 DNA (Dunn and Studier, 1983) into the *Bam*HI site so as to direct transcription in the counterclockwise orientation (opposing the tetracycline promoter). Plasmids pAR2156 and pAR2192, which have unique *Xba*I–*Bam*HI or *Bam*HI cloning sites, respectively, a short distance downstream from the start of T7 transcription, were constructed by A.Rosenberg, J.J.Dunn and F.W.Studier (unpublished data). Plasmid pKK3535 (Brosius *et al.*, 1981) containing the entire *rrn*B operon was obtained from Harry F.Noller, and a derivative of pKK3535 (pNO2680) having a unique *Kpn*I restriction site between the P1–P2 promoters and the 5′ end of 16S rRNA was kindly provided by Richard Gourse. *Sma*I was used to remove a 770-bp fragment from within the 16S rDNA region as previously described (Gourse *et al.*, 1982). Following cloning of DNA fragments and identification of recombinant clones by standard techniques, plasmids were moved into *E. coli* BL21(DE3), a λ lysogen of BL21 ($r_B^-m_B^-$*rif*^{S}) in which the prophage carries a copy of the gene for T7 RNA polymerase under control of the *lac* UV5 promoter (Studier and Moffatt, 1986).

Enzymes and linkers

Restriction endonucleases and enzymes used in cloning DNA were obtained from New England BioLabs. T7 RNA polymerase was purified from induced BL21/pAR1219 cells (Davenloo *et al.*, 1984) using an improved method; BL21 apparently lacks the protease responsible for nicking T7 RNA polymerase during purification that is present in most *E. coli* strains (J.J.Dunn, unpublished). Purified RNase III was the kind gift of Hugh D.Robertson. A *Bam*HI/*Kpn*I linker–adaptor (CGGATCCGGTAC) was synthesized using CED phosphoramidites (American BioNuclear) and a Microsyn 145 DNA synthesizer (Systec, Inc.).

In vitro transcription

Reaction mixtures contained 20 mM $NaPO_4$ at pH 7.7, 8 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine-HCl, 50 mM NaCl and 1 mM each of ATP, CTP, GTP and UPT. Labeled RNAs were synthesized by including [α-^{32}P]UTP in the reaction mixture. Template DNAs (~20 μg/ml) were linearized by cutting with an appropriate restriction endonuclease followed by phenol extraction and ethanol precipitation. Synthesis was initiated by addition of 5 μg/ml T7 RNA polymerase. RNAs were incubated with RNase III as previously described (Dunn and Studier, 1983).

Radiolabeling of plasmid-coded rRNAs in vivo

Strains of *E. coli* BL21(DE3) carrying the recombinant plasmids used in this study were grown at 37°C in 10 ml ZPM labeling medium supplemented with 0.2% casamino acids (Difco), 10% Luria broth (Gourse *et al.*, 1982) and 0.2% glucose. The *lac* UV5 promoter was routinely induced by adding IPTG to a final concentration of 0.5 mM when the growth rate of the culture began to slow down (late logarithmic growth). After an additional 45 min of shaking at 37°C, rifampicin (250 μg/ml) was added, followed at various times by addition of carrier-free $^{32}PO_4$ (2 μCi/ml) to label any RNAs that continued to be synthesized. Samples were labeled for 20 min.

Electrophoresis of ribosomes and plasmid-coded rRNAs

RNAs synthesized *in vitro* were analyzed by electrophoresis in 40 mM Tris/20 mM acetic acid/2 mM Na_3EDTA plus ethidium bromide (1 μg/ml) on a 1.25% agarose gel and were visualized by fluorescence or by autoradiography. The rRNAs and ribosomal particles produced *in vivo* were analyzed by electrophoresis in gels containing 3% polyacrylamide and 0.5% agarose as previously described (Gourse *et al.*, 1982). The rRNA content of electrophoretically resolved ribosomal particles was determined by electrophoresis in a second dimension followed by autoradiography (Stark *et al.*, 1982).

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