Processing of precursor ribosomal RNA and the presence of a modified ribosome assembly scheme in *Escherichia coli* relaxed strain

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Received 28 December 1984

An electrophoretic system capable of separating 25 S, 23 S, 17.5 S and 16 S ribosomal RNA (rRNA) species was used to study the synthesis and fate of rRNA during amino acid starvation and resupplementation of *E. coli* relaxed strain KL99. This *E. coli relA1* strain responded to an amino acid starvation by increasing the rate of synthesis of 25 S and 17.5 S precursor rRNA. When the limiting amino acid was resupplemented, a previously observed 40-fold increase in the cellular guanosine 5′-diphosphate, 3′-diphosphate content [Mol. Gen. Genet. (1983) 192, 5–9] appeared to cause a reduction in new rRNA synthesis. Following amino acid resupplementation, the precursor 25 S and 17.5 S rRNA accumulated during the amino acid starvation were conserved and processed to 23 S and 16 S rRNA species, respectively. This suggests that a modified ribosome assembly scheme involving stable precursor rRNA exists in *relA1* bacteria during periods of amino acid limitation and resupplementation.

Precursor rRNA *Processing* *Ribosome assembly*

1. INTRODUCTION

Amino acid starvation of stringent strains of *E. coli* (*relA*$^+$) results in the accumulation of cellular guanosine 5′-diphosphate, 3′-diphosphate (ppGpp) and an accompanying inhibition of RNA synthesis [1–4]. Relaxed strains (*relA1*), on the other hand, decrease their ppGpp content and continue to accumulate stable RNA in response to an amino acid starvation [5]. When the limiting amino acid is resupplemented to previously starved relaxed bacteria, basal level ppGpp increases while RNA accumulation decreases [5]. Rapid growth and protein synthesis also resume during the resupplementation period, and there is considerable interest in how relaxed bacteria continue to assemble ribosomes and grow, while RNA accumulation decreases. One possible way to accomplish this would be for cells to utilize rRNA (accumulated during the starvation) for ribosome assembly following amino acid resupplementation. Alternatively, rRNA accumulated during the starvation could be degraded and used to synthesize new rRNA which would be assembled into ribosomes following resupplementation.

We have investigated the fate of the ribosomal RNA species accumulated following amino acid starvation of an *E. coli relA1* strain, KL99. This strain was chosen because during amino acid starvation it is one of the *relA1* strains that exhibited a large increase in rRNA accumulation [5]. Our findings indicate that after amino acid resupplementation 25 S and 17.5 S precursor rRNA, synthesized during the starvation, were conserved and processed to 23 S and 16 S rRNA, respectively. The significance of these findings is discussed in relation to bacterial ribosome assembly and the regulation of rRNA synthesis by ppGpp.

2. MATERIALS AND METHODS

Carrier-free orthophosphoric acid $H_3{}^{32}PO_4$, [^{3}H]uridine, Formula 949, and Aquasol 2 scintillation fluid were obtained from New England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

Nuclear, Boston, MA. All other reagents were of analytical grade and obtained from local sources.

2.1. *Bacterial strains, media, and growth conditions*

An *E. coli* K-12 strain, KL99 (*Hfr PO42, thi-1, relA1, spoT1*), was used in this study [6]. Bacteria were grown at 37°C on Hershey's Tris minimal medium [7] containing 0.33 mM orthophosphate, 0.3% glucose, 2 μg/ml thiamine, and 50 μg/ml of each required amino acid. Amino acid starvation was initiated by the addition of 500 μg/ml of valine and was reversed by adding 500 μg/ml of isoleucine.

2.2. *RNA accumulation*

Extraction and quantitation of accumulated ^{32}P-labeled RNA was described previously [8]. All ^{32}P-labeled RNA was normalized and expressed as cpm per absorbance unit of bacterial culture at 720 nm. There are approximately 1×10^9 cells/A_{720}.

2.3. *Rate of rRNA synthesis*

Cultures were starved for isoleucine at zero time and resupplemented 41 min later. At various times 1-ml aliquots were pulse labeled for 2 min with 10 μCi [^{3}H]uridine, together with 0.2 μg uridine and 100 μg cytidine and labeling was stopped by the addition of 5 ml of ice-cold medium. One ml of ice-cold starved carrier cells and 50000 cpm of ^{32}P-labeled starved cells were added to each sample as an internal standard. Cells were harvested and lysed by a lysozyme-deoxycholate, freeze-thaw procedure described previously [5]. Aliquots were loaded onto composite 3% acrylamide–0.3% agarose slab gels [9] that contained 0.089 M Tris (pH 8.3), 0.089 M boric acid, 2.5 mM EDTA and 1% SDS. In order to adequately separate rRNA species, the gels were run at 9 V/cm for ½ h after the marker dye had run off the end of the gel. rRNA was visualized by staining with ethidium bromide (2 μg/ml in 0.5 M potassium acetate) for ½ h and 25 S, 23 S, 17.5 S, and 16 S rRNA species were cut out of the gel and immersed in 0.3 ml of 30% hydrogen peroxide in scintillation vials. The vials were sealed and placed at 100°C for 10 min followed by 12 h at 50°C. After cooling, 15 ml of Aquasol 2 scintillation fluid was added and each vial was counted. After samples were adjusted for the internal ^{32}P standard, the amount of [^{3}H]uridine per min per A_{720} was used as a measure of the rate of synthesis of each RNA species.

2.4. *Fate of accumulated RNA*

Bacteria were grown to an A_{720} of about 0.4 at which time isoleucine starvation was initiated by the addition of 500 μg/ml of valine. Five min later 10 μCi/ml [^{3}H]uridine, together with 3 μg/ml uridine and 100 μg/ml cytidine were added to the culture. The starvation was maintained for 40 min at which time 200 μg/ml cold uridine was added to half the culture and the remainder was left unchased. Two min later, the starvation was relieved by adding 500 μg/ml of isoleucine. Aliquots were withdrawn at various times and treated as described above for the rate of rRNA synthesis. Samples were adjusted for the internal ^{32}P standard and the amount of [^{3}H]uridine per A_{720} was determined for each rRNA species.

The procedure of Tokimatsu et al. [10] was used for the isolation of 50 S and 30 S ribosomal subunits. Briefly, samples were lysed and treated as described above with the following exceptions: cells were rendered 0.1% in sodium deoxycholate; no SDS was added to the samples; the lysate was centrifuged at 20000 × *g* for 15 min; and aliquots were taken from the supernatant for electrophoresis. Samples were applied to composite slab gels of 2.75% acrylamide and 0.5% agarose in 25 mM Tris, 2 mM $MgCl_2$ and electrophoresed at 50 mA for 3 h in the same reservoir buffer. 50 S and 30 S subunits were stained, cut out, and treated as described above.

3. RESULTS

We have previously analyzed the rRNA accumulated in *E. coli relA1* strain KL99 during isoleucine starvation and its subsequent resupplementation by electrophoresis on 3% polyacrylamide–0.3% agarose composite slab gels [5]. Since this system could not separate 25 S and 23 S rRNA, only the accumulation of 17.5 S and 16 S rRNA was considered at that time. We observed that 17.5 S precursor rRNA was stabilized during the amino acid starvation, presumably by attaching to fast sedimenting materials (e.g., membranes). Following amino acid resupplementation, 17.5 S precursor rRNA appeared to be

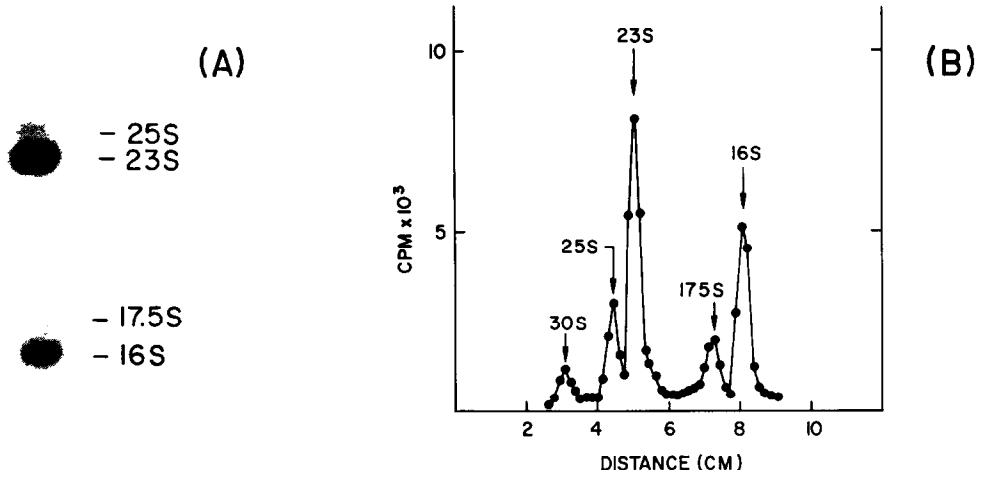

Fig.1. rRNA gel electrophoresis. (A) Autoradiogram. A culture of KL99 was grown in Hershey's Tris minimal medium [7] with 0.33 mM phosphate and was labeled with $H_3{}^{32}PO_4$ (1.5 μCi/ml) for two generations. 500 μg/ml of valine was added to the culture at zero time and a sample was removed 30 min later and lysed as described in section 2. The RNA in these extracts was then separated electrophoretically in 3% acrylamide–0.3% agarose composite gel in the presence of 1% SDS. The gel was covered with saran wrap and directly exposed with a Kodak XR-5 X-ray film. (B) Counting of separated rRNA species. *E. coli* was grown and starved as above. 5 min after the starvation, [^{3}H]uridine (10 μCi/ml) together with 1.6×10^{-5} M cold uridine and 3.5×10^{-4} M cytidine were added to the culture. Samples were removed 30 min later, lysed and rRNA was electrophoretically separated as described in (A). The gel lane was sliced into 2 mm sections and counted as described in section 2. cpm were plotted as a function of the distance migrated.

released from the fast sedimenting material and either degraded or processed to 16 S rRNA.

The addition of 1% SDS to the rRNA gel electrophoresis system of Peacock and Dingman [9] has allowed us to resolve 25 S, 23 S, 17.5 S and 16 S rRNA species [11]. Fig.1 shows that when *E. coli* KL99 was starved for isoleucine both precursor 25 S and 17.5 S rRNA were accumulated. The separation of different rRNA species enables us to measure their rates of synthesis during amino acid starvation and resupplementation. Fig.2 illustrates that immediately after initiating isoleucine starvation the rate of synthesis of precursor 25 S and 17.5 S rRNA increased about 100 and 70%, respectively, and the rate of synthesis remained elevated throughout the entire starvation period. On the other hand, during the same starvation period the rate of synthesis of 23 S and 16 S rRNA increased only 40 and 20%, respectively. Interestingly, when the limiting amino acid was resupplemented, there was a very substantial decrease in the rate of synthesis of all rRNA species measured (fig.2).

A decrease in the rate of synthesis of 23 S and 16 S rRNA during the resupplementation period could pose a problem for continued ribosome assembly. One way to resolve this dilemma is for the cells to conserve the precursor rRNA synthesized during the amino acid starvation and process them during the resupplementation period. Consequently, we have determined the fate of the rRNA accumulated during amino acid starvation by labeling cultures with [^{3}H]uridine and assaying the cultures after a uridine chase and subsequent amino acid resupplementation. Fig.3 shows that the amount of radioactivity in precursor 25 S and 17.5 S rRNA was elevated at the end of the amino acid starvation. Following resupplementation, there was a decrease in the amount of labeled precursor rRNA (after a short lag period). Conversely, levels of labeled 23 S and 16 S rRNA, which were diminished at the end of the starvation, increased during the resupplementation period after a similar lag period. From 7 to 22 min after resupplementation there was a nearly equal decrease in precursor 25 S and 17.5 S rRNA and

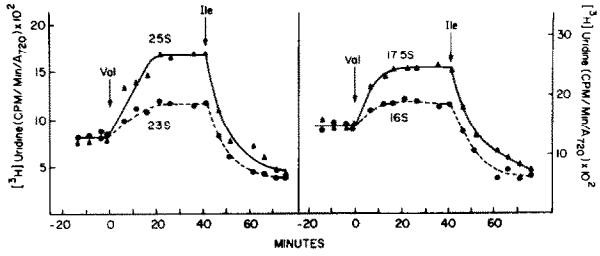

Fig.2. The rate of rRNA synthesis in KL99. Cells were grown, and starved as in fig.1A except that no $H_3{}^{32}PO_4$ was added to the culture. 41 min after initiating isoleucine starvation, the starvation was relieved by the addition of 500 μg/ml of isoleucine. To determine the rate of rRNA synthesis, 1 ml samples were removed from the culture flask at various times and pipetted into tubes containing [^{3}H]uridine (20 μCi/ml), 0.8×10^{-6} M cold uridine and 3.5×10^{-4} M cytidine. Labeling was stopped 2 min later. One ml of ice-cold starved carrier cells and 50000 cpm of ^{32}P-labeled starved cells were added to each sample as an internal standard. Samples were lysed and rRNA was electrophoresed in 3% acrylamide–0.3% agarose composite slab gels containing 1% SDS. After locating the various rRNA species, the rate of rRNA synthesis was determined from the counts incorporated into individual rRNA species. Left: 25 S (▲), 23 S (●); right: 17.5 S (▲), 16 S (●).

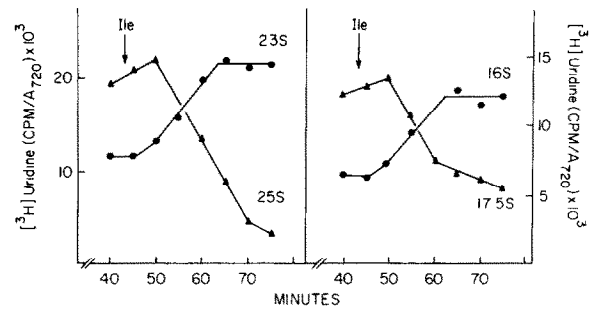

Fig.3. Fate of 25 S, 23 S, 17.5 S and 16 S RNA in KL99. Cells were grown on Hershey's Tris minimal medium [7] and starved for isoleucine by the addition of valine at zero time as in fig.1. 5 min after valine addition 10 μCi/ml [^{3}H]uridine together with 1.6×10^{-5} M cold uridine and 3.5×10^{-4} M cytidine were added to the culture. A uridine chase (final concentration 1×10^{-3} M) was added at 41 min and 2 min later, half of the culture was resupplemented with 500 μg/ml isoleucine. Samples were treated as described in fig.2 and cpm in each rRNA species were determined. Left: 25 S (▲), 23 S (●); right: 17.5 S (▲), 16 S (●).

an increase in 23 S and 16 S rRNA, respectively. 23 S and 16 S rRNA reached a new steady state level at this point, but the cellular content of precursor rRNA continued to decrease to low levels. In control experiments we demonstrated that the uridine chase alone did not cause processing of precursor rRNA and that existing precursors were still processed when the culture was resupplemented but not chased [11].

If cells compensate for decreased rRNA synthesis during the resupplementation period by utilizing the existing precursor rRNA, then RNA synthesized during the starvation should enter ribosomal subunits following resupplementation. To investigate this possibility, cells were starved for isoleucine and labeled with [^{3}H]uridine throughout the starvation. Aliquots of culture were removed before and after a uridine chase and subsequent amino acid resupplementation. At each time point, 50 S and 30 S ribosomal subunits were separated on non-denaturing slab gels [10]. Fig.4 shows that following a uridine chase and amino acid resupplementation there was a large increase in the amount of radioactivity that comigrated with 50 S and 30 S ribosomal subunits. A chased

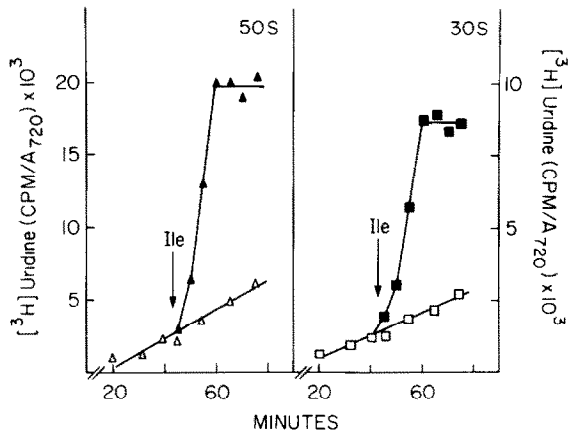

Fig.4. RNA associated with 50 S and 30 S ribosomal subunits of KL99. Cells were grown, starved, labeled and resupplemented as in fig.3. 50 S and 30 S ribosomal subunits were extracted and isolated electrophoretically on nondenaturing 2.75% acrylamide–0.5% agarose composite slab gels [10]. The radioactivity associated with each ribosomal subunit was counted as described in section 2. Left: 50 S, chased (△), chased and resupplemented (▲); right: 30 S, chased (□), chased and resupplemented (■).

(but not resupplemented) control demonstrated that the uridine chase alone did not cause increased incorporation of [^{3}H]uridine in the 50 S and 30 S fractions. These data suggest that rRNA synthesized during the starvation are conserved and enter ribosomal subunits following amino acid resupplementation. Hence, the accumulation of RNA during an amino acid starvation provides the necessary rRNA for increased ribosome assembly and cell growth when the starvation is relieved.

4. DISCUSSION

It has been widely accepted that ribosome assembly is coupled to the synthesis of ribosomal RNA [12,13]. However, ribosome assembly which is exclusively coupled to transcription does not explain the above findings. Our results indicate that synthesis and processing of rRNA can be separated during the amino acid starvation and resupplementation of *E. coli relA1* strain KL99. This suggests that transcription may be uncoupled from ribosome assembly during the initial period of amino acid resupplementation. In addition, it implies that assembly of ribosomes by the concomitant synthesis and processing of rRNA may be the result of the efficiency of the metabolic processes associated with balanced growth, rather than a requirement of the assembly process.

Using this modified ribosome assembly scheme, *E. coli* KL99 accumulates and maintains excess precursor rRNA during an amino acid starvation [5]. After amino acid resupplementation, the rate of synthesis of rRNA decreases (fig.2) and the previously accumulated 25 S and 17.5 S precursor rRNA are processed to 23 S and 16 S rRNA, respectively (fig.3). In fact, processing of precursor rRNA during the resupplementation period results in a previously observed increase in the amount of 16 S rRNA [5] while the rate of synthesis of new 16 S rRNA actually decreases (fig.2). To adjust for the decreased rate of new rRNA synthesis, accumulated RNA leave a fast sedimenting fraction [5] and comigrate with 50 S and 30 S ribosomal subunits following amino acid resupplementation (fig.4). The relative stability of precursor rRNA accumulated during amino acid starvation and their apparent conservation during the initial resupplementation period, might allow bacteria to rapidly increase their ribosome content while the rate of rRNA synthesis decreases.

Immediately following amino acid starvation there is a short lag which precedes the processing of precursor rRNA (fig.3) and the appearance of label in ribosomal subunits (fig.4). The lag may be the result of a requirement for protein synthesis which resumes following amino acid resupplementation [5]. Hence, the lag is consistent with an RNA processing and ribosome assembly scheme which requires new protein synthesis [12,14–17].

The increase in new rRNA synthesis during the starvation and the subsequent restriction of rRNA synthesis following resupplementation appear to be regulated by changes in cellular ppGpp content. In previous studies with KL99, we have demonstrated an inverse correlation between the rate of rRNA synthesis and ppGpp content [19]. The reduction in the rate of rRNA synthesis following amino acid resupplementation is most likely caused by a previously observed 40-fold increase in cellular ppGpp content during this period [5,19]. This reduced rate of rRNA synthesis may force the cell to process existing precursor rRNA which are abundant. Similar control of rRNA synthesis by ppGpp may also exist during other shifts from slow to rapid rates of growth. In particular, cells grown at 23°C maintain increased amounts of RNA and low ppGpp basal levels [18]. When these cells are shifted to 40°C they respond by temporarily restricting RNA synthesis and increasing ppGpp content [19]. Although the stability and fate of the RNA synthesized at 23°C remain to be determined, the similarity is clear and implies that this modified ribosome assembly scheme may be common during growth imbalances.

ACKNOWLEDGEMENT

This work was funded by grants from Temple University Bio-Med Fund and Grant-in-Aid of Research.

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