Reconstitution of 50 S ribosomal subunits from *Bacillus stearothermophilus* with 5 S RNA from spinach chloroplasts and low-M_r RNA from mitochondria of *Locusta migratoria* and bovine liver

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Reconstitution experiments with 50 S ribosomal subunits from *Bacillus stearothermophilus* demonstrate that spinach chloroplast 5 S rRNA can be incorporated into the bacterial ribosome and yield biologically active particles, thereby establishing the eubacterial nature of chloroplast 5 S rRNA. In contrast, mitochondria from *Locusta migratoria* or bovine liver do not appear to contain discrete, low-M_r RNAs, which can replace 5 S rRNA in the functional reconstitution of *B. stearothermophilus* ribosomes.

Chloroplast 5 S rRNA *Mitochondrial 5 S rRNA* *50 S ribosome* *Reconstitution* *Protein synthesis* *Evolution*

1. INTRODUCTION

Reconstitution experiments on bacterial 50 S ribosomal subunits without 5 S rRNA have demonstrated the importance of this molecule for the function of these subunits. Incorporation of 10 different prokaryotic 5 S rRNAs tested led to active *Bacillus stearothermophilus* 50 S subunits [1,2]. Since several workers [3,4] have found that ribosomes from organelles have bacterial characteristics, as proposed by the endosymbiotic hypothesis [5,6], it is interesting to examine whether a 5 S rRNA molecule from organelle ribosomes can be incorporated into a bacterial ribosome. Recent results from comparative structural analysis experiments demonstrated the eubacterial character of the chloroplast 5 S rRNA from *Spinacea oleracea* ribosomes [7]. We demonstrate here that the chloroplast 5 S ribosomal RNA can function in the eubacterial 50 S subunit from *B. stearothermophilus*.

Mitochondrial ribosomes from higher plants contain 5 S rRNA [8], but no such molecule has yet been detected in mitochondrial ribosomes from fungi or animals [6,9] nor have sequences coding for 5 S RNA-like molecules been identified in animal mitochondrial DNA [10,11]. However, since the ribosomal RNAs from animal mitochondria have lower M_r values it was thought that a molecule equivalent to 5 S rRNA, but of proportionately smaller size, may be hidden in the mitochondrial tRNA fraction in preparations of mitochondrial RNA. The so-called 3 S_E RNA from hamster mitochondria was proposed to be such an equivalent [12], but was later shown to be an unusual $tRNA^{Ser}$ species, which does not possess the dihydrouridine loop [13]. With ribosomal reconstitution experiments we attemp-

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ted to identify a 5 S rRNA equivalent in the bulk tRNA fraction of mitochondrial RNA from *Locusta migratoria* and bovine liver, and found with this technique no evidence to support the existence of a separate '5 S rRNA-like' molecule in these two organisms.

2. EXPERIMENTAL

B. stearothermophilus 799 was grown at 61°C to early-log phase in medium L (10 g tryptone, 5 g yeast extract, 5 g NaCl, 5 g glucose, and 990 μg $MnCl_2$, per l H_2O). The pH was adjusted to 7.0 with NaOH. The preparation and separation into subunits of ribosomes were as in [14]. 23 S rRNA was isolated by phenol extraction from 70 S ribosomes [15] and centrifugation in a 10–30% sucrose gradient containing 20 mM Tris–HCl (pH 7.6), 50 mM KCl and 1% methanol, in a Beckman SW 28 rotor at 25000 rpm and 4°C for 24 h.

5 S rRNA was purified by preparative gel electrophoresis on 12% polyacrylamide slab gels in 7 M urea. After localization of the 5 S rRNA band through UV-shadowing, the band was excised, and incubated in buffer (0.5 mM ammonium acetate, 0.01 M Mg-acetate, 0.1 mM EDTA, 0.1% SDS) at 30°C for 10 h. This procedure was repeated and the 5 S rRNA was precipitated with ethanol at −20°C for several hours. After centrifugation the 5 S rRNA was resuspended in water and stored at −80°C.

Chloroplast 5 S rRNA from *S. oleracea* ribosomes was isolated as in [7]. RNA from bovine liver mitochondria [16] was prepared by SDS-phenol extraction and centrifugation on 10–30% sucrose gradients (containing 0.5% SDS, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4) to separate the rRNA from the tRNA fraction. Fractions containing the 3–4 S RNA peak were pooled, precipitated with ethanol and used as the bulk tRNA fraction from bovine liver mitochondria.

The preparation of the RNA fraction from *L. migratoria* was performed as in [17].

Total ribosomal proteins from 50 S subunits (TP50) from *B. stearothermophilus* were prepared as in [18], except that acetone-precipitated proteins were dissolved in Rec-4 buffer (20 mM Tris–HCl (pH 7.4), 4 mM Mg-acetate, 400 mM NH_4Cl, 4 mM β-mercaptoethanol, 0.2 mM EDTA) containing 6 M urea, dialyzed overnight against a 500-fold volume of the same solution, once for 45 min against a 100-fold volume of Rec-4 buffer and once again for 35 min against a 100-fold volume of Rec-4 buffer. Precipitated proteins were removed by centrifugation, and the supernatant stored in small portions at −80°C. The two-step reconstitution procedure was as in [18,19], except that the volume of the assay was 50 μl containing 1.25 A_{260} 23 S rRNA, 1.25 e.u. TP50 (1 e.u. of protein is the amount extracted from 1 A_{260} unit of 50 S subunits) and various amounts of 5 S rRNA or tRNA, under Rec-4 conditions; the temperature in the second reconstitution step was raised to 60°C. After reconstitution 40 μl of the reconstitution assay (corresponding theoretically to 1.0 A_{260} 50 S subunits) were tested in a poly(U)-directed polyphenylalanine system [20] containing 0.7 A_{260} 30 S subunits from *E. coli* A19.

To determine the extent of incorporation of 5 S rRNA or tRNA into 50 S subunits we used either polyacrylamide gel electrophoresis of phenol-extracted RNA from reconstituted particles, or centrifugation in a 10–30% sucrose gradient [in 10 mM Tris–HCl (pH 7.4), 0.3 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol] of particles reconstituted with ^{32}P-labelled mitochondrial low-M_r RNA (tRNA fraction). The centrifugation was carried out in a Beckman SW 28 rotor at 20000 rpm and 4°C for 16 h. The 5′-^{32}P labelling of the RNA was as in [21].

3. RESULTS AND DISCUSSION

In the first experiments, we investigated the incorporation of chloroplast ribosomal 5 S rRNA from *S. oleracea* into 50 S ribosomal subunits of *B. stearothermophilus*. Fig.1 presents the activity of reconstituted *B. stearothermophilus* 50 S subunits in a poly(U)-dependent polyphenylalanine-synthesizing system as a function of the chloroplast ribosomal 5 S rRNA concentration. The activity of particles lacking 5 S rRNA was approx. 4%, and the maximal biological activity (75%) was reached with a 5 S rRNA:23 S rRNA ratio of 1:1, reflecting the stoichiometry of these molecules in active ribosomes [23]. The chloroplast ribosomal 5 S rRNA molecule apparently has sufficient structural and functional similarities to the usual 5 S rRNA component of *B. stearothermophilus* ribosomes to support protein synthesis in our system.

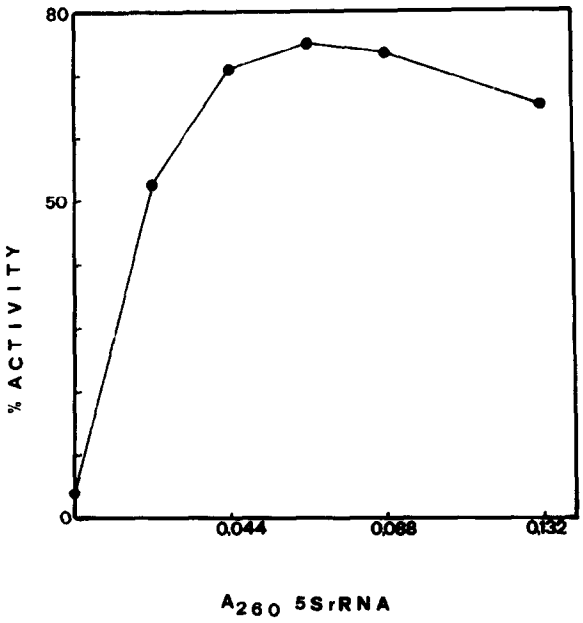


Fig.1. Activity of reconstituted *B. stearothermophilus* 50 S subunits in a poly(U)-dependent polyphenylalanine-synthesizing system as a function of *S. oleracea* chloroplast 5 S rRNA concentration. 100% is defined as the activity of native 50 S subunits from *B. stearothermophilus* in polyphenylalanine synthesis, and 0% as the activity of a reconstitution assay without 5 S rRNA.

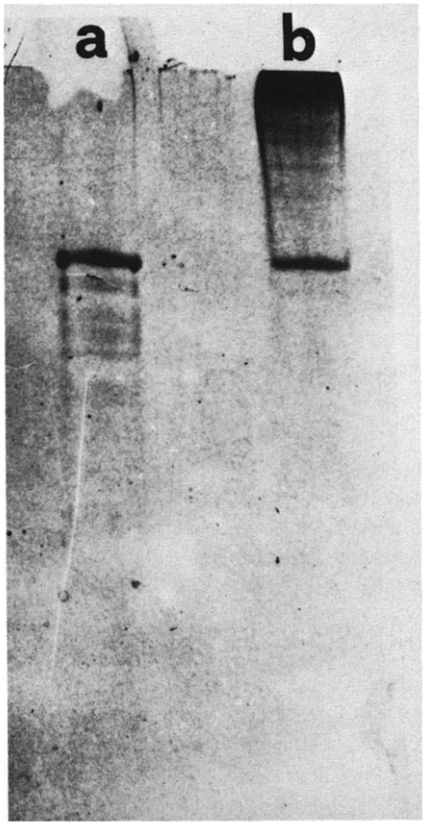


Fig.2. Urea (7 M)/polyacrylamide (12%) gel electrophoresis of *S. oleracea* chloroplast 5 S rRNA used in the reconstitution (a) and extracted from reconstituted purified 50 S *B. stearothermophilus* subunits (b). 23 S rRNA is too large to migrate into the 12% gel under these conditions. Gel dimensions: 16 × 14 × 0.1 cm.

To determine whether the chloroplast 5 S rRNA was actually incorporated into the 50 S ribosomal subunit of *B. stearothermophilus* we analyzed the rRNA extracted from reconstituted subunits (fig.2). As seen in fig.2 (lane b), the reconstituted particles do contain chloroplast 5 S rRNA, indicating that this heterologous 5 S rRNA retains a high degree of structural and functional similarity to eubacterial 5 S rRNA. This observation can probably be extended to all chloroplast 5 S rRNAs which have been sequenced, since nearly all their primary sequences show extremely strong homologies (95–100%) [7].

In the following experiments we used the above approach in attempts to identify a 5 S rRNA homologue in animal mitochondria. Such molecules have not yet been detected in isolated animal mitochondrial ribosomes, possibly because they are lost during preparation of the ribosomes.

Reconstitution experiments could be helpful in detecting such a molecule using the bulk mitochondrial tRNA fraction extracted from *L. migratoria* and bovine liver mitochondria as a source of the 5 S rRNA homologues.

As shown in fig.3 addition of increasing amounts of bulk mitochondrial tRNA to the reconstitution mixture does not stimulate polyphenylalanine synthesis. However, commercial bulk tRNA from *E. coli*, which contains traces of 5 S rRNA, does stimulate this activity. Clearly, there is no molecule in the mitochondrial bulk tRNA fractions that can substitute for eubacterial 5 S rRNA in the functional reconstitution assay.

To eliminate the possibility that an inactive molecule present in the preparation was incorporated, we labelled the bulk mitochondrial tRNA with ^{32}P at the 5′-terminus (fig.4), reconstituted

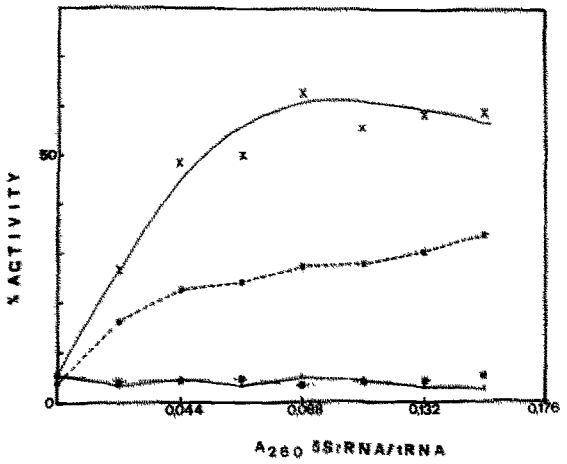


Fig.3. Activity of reconstituted *B. stearothermophilus* 50 S subunits in a poly(U)-dependent polyphenylalanine-synthesizing system as a function of bulk mitochondrial tRNA concentration from bovine liver (■···■), *L. migratoria* (▲—▲), commercial *E. coli* MRE 600 (●– – –●), and 5 S rRNA from *E. coli* (×—×). For the definition of % activity see legend to fig.1.

and analyzed the reconstituted particles by sucrose gradient centrifugation. Fig.5a shows that there is no radioactivity in the reconstituted 50 S particle. All radioactively labelled mitochondrial tRNAs are at the top of the gradient. We obtained the same result using 5′-^{32}P-labelled bulk *B. stearothermophilus* tRNA from which 5 S rRNA had been removed by Sephadex G-100 chromatography [23] (fig.5b). This result further supports the conclusion that the activity of reconstituted 50 S particles with commercial *E. coli* tRNA (fig.3), can be ascribed to the contamination with 5 S rRNA. An examination of the integrity of the labelled rRNAs used on a polyacrylamide gel (fig.6) indicated no degradation. These results agree well with the lack of polyphenylalanine synthesis activity of such reconstituted particles.

In summary, we conclude that spinach chloroplast 5 S rRNA is of eubacterial origin, and our failure to demonstrate a '5 S rRNA-like' molecule in mitochondrial ribosomes from *L. migratoria* and bovine liver suggests that such molecules are either absent from animal mitochondrial ribosomes, or occur in such an altered structure that then cannot be incorporated into ribosomes of the eubacterial type. Another intriguing possibility is that a fragmentary 5 S rRNA sequence has become part of the large ribosomal subunit RNA as suggested in [24].

Fig.4. Urea (7 M)/polyacrylamide (12%) gel electrophoresis of 5′-^{32}P-labelled bulk mitochondrial tRNA from *L. migratoria* (a) and bulk tRNA from *B. stearothermophilus* (b). Material contained within the arrows was eluted and used for reconstitution experiments. Gel dimensions: 20 × 13 × 0.1 cm.

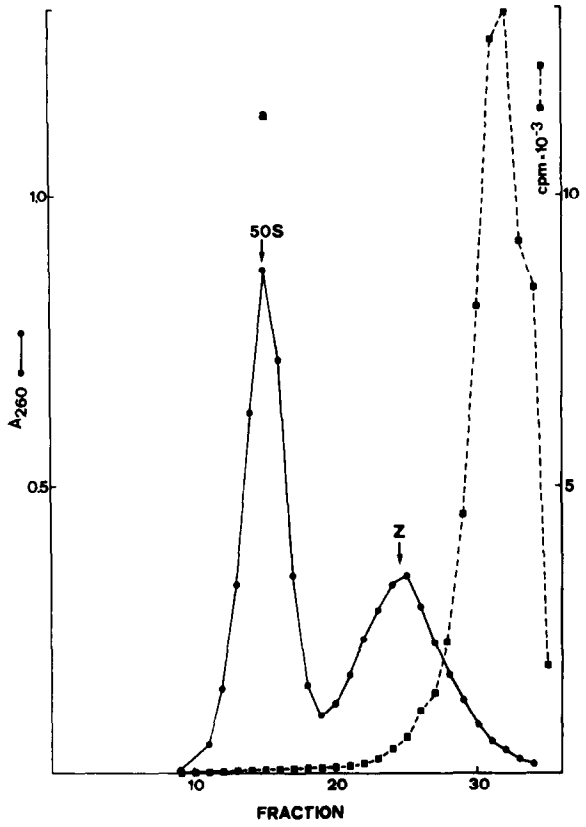
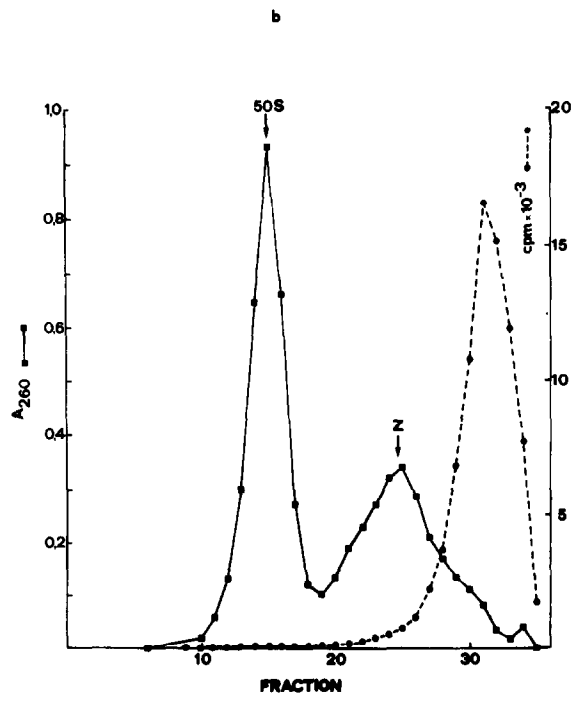


Fig.5. Sucrose gradients of *B. stearothermophilus* 50 S subunits reconstituted with 5′-^{32}P-labelled bulk mitochondrial tRNA from *L. migratoria* (a) and 5′-^{32}P-labelled bulk tRNA from *B. stearothermophilus* (b). Peak z in (a) and (b) presumably contains 16 S rRNA and incompletely assembled particles. The 50 S subunit peak was identified by comparison to control gradient profiles of native 50 S subunits.



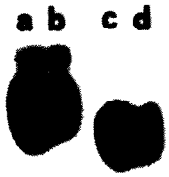


Fig.6. Urea (7 M)/polyacrylamide (12%) gel electrophoresis of 5′-^{32}P-labelled bulk tRNA: (a) *B. stearothermophilus* bulk tRNA extracted from fraction 32 of the sucrose gradient shown in fig.5b; (b) *B. stearothermophilus* bulk tRNA; (c) *L. migratoria* mitochondrial bulk tRNA extracted from fractions 31 and 32 of the sucrose gradient shown in fig.5a; (d) *L. migratoria* mitochondrial bulk tRNA. Gel dimensions: 20 × 13 × 0.1 cm.

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