1993

RECONSTITUTION OF A MINIMAL SMALL RIBOSOMAL SUBUNIT

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INTRODUCTION

It is appropriate that at a meeting dedicated to H. G. Wittmann we should emphasize comparative studies of the three-dimensional structure of the ribosome since he and his collaborators have made such important contributions to this field. In this paper we present data detailing the first isolation of small mitochondrial ribosomal subunits from the hemoflagellate *Leishmania tarentolae*. Their structure is interesting because these are the smallest ribosomes yet found (their small subunit rRNA sediments at 9S and is only 610 nucleotides long). We also show that particles similar in structure to these small subunits can be reconstituted from *in vitro* transcribed mitochondrial 9S rRNA and *E. coli* proteins.

The 9S and 12S ribosomal RNAs (rRNAs) are the major RNA components of the mitochondrion (kinetoplast) of *Leishmania tarentolae* (Simpson and Simpson, 1978). It has been proposed that the 9S RNA is the small kinetoplastid ribosomal subunit rRNA (de la Cruz *et al.*, 1985a) and the 12S RNA is the large kinetoplastid ribosomal subunit rRNA (de la Cruz *et al.*, 1985b). These assignments were based on the strong similarities between the secondary structures of these RNAs with the consensus small and large subunit rRNA secondary structures and on the occurrence of essential conserved sequences, such as the 520, 720 and 1400 regions (*E. coli* numbering system). However, the existence of kinetoplast ribosomes has not been directly demonstrated.

To test whether 9S RNA can form small subunits, we attempted to reconstitute the small ribosomal subunit (Traub *et al.*, 1971) from heterologous components, *Leishmania tarentolae* 9S RNA and *E. coli* small subunit ribosomal proteins. Heterologous reconstitutions have been previously achieved when the rRNA and ribosomal proteins are from different bacteria (Nomura *et al.*, 1968; Higo *et al.*, 1973; Goldberg and Steitz, 1974; Held *et al.*, 1974). Recently, *in vitro* T7 transcripts of wild type and mutant *E. coli* 16S rRNA have also been reconstituted with *E. coli* ribosomal proteins (Krzyzosiak *et al.*, 1987; Melancon *et al.*, 1987; Scheinman, 1989).

We show in this paper that hybrid subunits can be reconstituted using *in vitro*

transcribed 9S RNA and *E. coli* ribosomal proteins. We also show that a 9S- and 12S-containing kinetoplast-mitochondrial fraction contains ribosomes and ribosomal subunits. The smaller subunit possesses the classical ribosomal structures of the platform, head and base, as do the hybrid reconstituted 9S subunits. This indicates that the 9S rRNA can be assembled *in vivo* and *in vitro* into ribosomal like subunits.

MATERIALS AND METHODS

In Vitro Transcription of 9S RNA

The 9S DNA sequence was amplified in a PCR reaction using plasmid pLt120 (Masuda *et al.*, 1979; de la Cruz *et al.*, 1984) as the template and primers corresponding to positions -28 to +4 (5'-primer) and 575 to 611 (3'-primer) of the 9S DNA and flanking sequence. The 5'-primer also contained a T7 polymerase promoter sequence, and an EcoRI site at its 5'-end. The 3'-primer contained a HindIII site at its 5'-end. PCR conditions used were described elsewhere (Scheinman, 1989). The PCR reaction was run on a 0.8% agarose gel, the appropriate band was excised, and the DNA was recovered by GeneClean. Approximately 1 microgram of this DNA was used for an *in vitro* transcription (Scheinman, 1989), with a final volume of 1 ml.

Reconstitutions

RNA was obtained from transcription reactions by phenol extraction and ethanol precipitation. After resuspension this RNA was combined with the appropriate amount of *E. coli* small ribosomal subunit proteins and reconstituted as described (Scheinman, 1989; Held *et al.*, 1973).

Sucrose Gradient Analyses and Electron Microscopy

Reconstituted subunits were concentrated by ultracentrifugation in a Beckman SW50.1 rotor at 41,000 RPM (157,000 x g) for 3.5 hrs at 4°C, resuspended in 200 microliters of 10 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM DTT, 0.5 mM EDTA and loaded onto sucrose gradients that were 20-30% sucrose in the same buffer. These gradients were centrifuged for 40 minutes at 50,000 RPM (220,000 x g) at 4°C in a Beckman VTi65 rotor, after which they were fractionated and monitored through an ISCO UA-5 continuous absorbance monitor. Subunits were collected and concentrated by ultracentrifugation, resuspended to a final concentration of 0.2 A_{260} units/ml, negatively stained by the double-layer carbon method (Lake, 1979), and visualized with a Philips 400 electron microscope.

Preparation of L. tarentolae Kinetoplast Small Subunits

L. tarentolae (UC strain) were grown as previously described (Braly *et al.*, 1974). Mid-log-phase cells were harvested by centrifugation and the kinetoplast-mitochondrial fraction was isolated by flotation in Renografin density gradients (Braly *et al.*, 1974; Simpson and Braly, 1970). Kinetoplast ribosomes were isolated by a modification of the method of Spithill *et al.* (1979).

The isolated kinetoplast fraction was resuspended in 125 mM sucrose, 10 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, and an equal volume of 2X protein synthesis buffer was added (50 mM Tris-HCl (pH 6.7), 20 mM $MgCl_2$, 300 mM KCl, 20 mM KH_2PO_4, 4 mM ATP, 10 mM ketoglutarate). The resulting solution was incubated for 10 minutes

at 25°C, and then 0.01 volume 5 mg/ml puromycin was added. The solution was incubated for three minutes at 25°C, diluted with 4 volumes 250 mM sucrose, 20 mM Tris (pH 7.9), 3 mM $MgCl_2$, and centrifuged at 10,000 x g for 15 minutes. The pellet was resuspended in 200 mM sucrose, 200 mM Tris-HCl (pH 8.0), 35 mM $MgCl_2$, 400 mM KCl, 25 mM EGTA, 2% Triton X-100, 40 mg heparin, the solution was homogenized and then clarified by centrifugation at 10,000 x g for 15 minutes. The solution was then layered on a 15-30% linear sucrose gradient made in the same buffer and centrifuged for 22 hr at 16,000 RPM at 4°C in a Beckman SW27 rotor. RNA was isolated from the gradient fractions by SDS lysis and phenol-chloroform extraction and ethanol precipitation as previously described (Simpson and Simpson, 1978).

Figure 1. Sucrose gradient sedimentation analysis of reconstituted hybrid small ribosomal subunits (*in vitro* transcribed *L. tarentolae* 9S RNA plus *E. coli* r-proteins) run on a 15-30% gradient. The peak labeled "20S" contains reconstituted particles that, by their migration, rRNA composition and structure, correspond to mitochondrial small subunits. Sedimentation is from left to right and the vertical scale measures absorbance at 254 nm. A 4% PAGE gel is shown at the left. Lanes were loaded with T7 *in vitro* transcribed 16S rRNA (lane 1), native 16S rRNA (lane 2), rRNA extracted from the 20S peak of the adjacent gradient (lane 3 -- contrasted to show a weak band), T7 *in vitro* transcribed 9S rRNA (lane 4), and 9S rDNA used for the transcription (lane 5). RNA was visualized by Ethidium Bromide/UV.

RESULTS AND DISCUSSION

Figure 1 shows the results of a sucrose gradient size fractionation of the reconstituted products. Two peaks are present. The major peak, sedimenting at 20S (labelled 20S), contains ribosomal subunit with a unique morphology. The leading peak consists of unreconstituted components. The position of *E. coli* small subunits in control experiments (labeled "30S") is shown in the figure.

To characterize the 20S particles, the 20S peak was collected from sucrose gradients, concentrated by ultracentrifugation, and either loaded onto polyacrylamide gels for electrophoresis or negatively stained for electron microscopy. Gel electrophoresis (shown at the left in Figure 1) indicated that the 20S peak contained 9S rRNA and did not contain 16S rRNA [possibly introduced with the small subunit proteins]. The left

two lanes contain 16S rRNA (*in vitro* and *in vivo* transcribed in lanes 1 and 2, respectively), lane 3 contains RNA extracted from the 20S peak, lane 4 contains *in vitro* transcribed 9S rRNA, and lane 5 contains 9S rDNA. The occurrence of a 9S band in the 20S fraction and the absence of detectible 16S in this peak indicates that the 9S rRNA has reconstituted into the 20S particles, and that the 20S particles are not partially folded 30S subunits.

Figure 2. Electron microscopic field of purified mitochondrial small ribosomal subunits. Electron microscopy is described in reference 24. The scale bar represents 25 nanometers. The electron micrograph of *L. tarentolae* ribosomes and subunits was obtained after partial purification on sucrose gradients of the peak shown in Figure 4. Individual small subunits from this preparation are shown in Figure 3B. A single eukaryotic cytoplasmic small subunit is enclosed in brackets and provides a useful size comparison. Mitochondrial ribosomes and large subunits are indicated by 'L' and 'M', respectively. Small subunits are indicated by arrows.

Figure 3. Gallery of representative views of (A) reconstituted hybrid small subunits (*in vitro* transcribed *L. tarentolae* 9S RNA, *E. coli* ribosomal proteins), (B) *L. tarentolae* small subunits, and (C) reconstituted wild-type (*E. coli* 16S rRNA, *E. coli* ribosomal proteins) small subunits. Subunits are shown in the "asymmetric projection" in the first three columns on the left, in the corresponding enantiomorphic projection in the middle three columns, and in the "quasi-symmetric" projection in the two columns on the right (Lake, 1976). Images of reconstituted hybrid- and purified mitochondrial- small subunits and ribosomes were obtained from the fractions in Figures 1 and 4.

Electron microscopy of the 20S peak disclosed ribosomal subunits which, although related to 30S subunits, were clearly smaller and had altered profiles. Figure 3A shows representative projections of these smaller particles, which we tentatively identify as hybrid small subunits. Subunits are shown in the "asymmetric projection" (Lake, 1976) in the first three columns on the left, and in the corresponding enantiomorphic projection in the second three columns. For comparison the corresponding profiles of reconstituted *E. coli* small subunits are shown in Figure 3C. The ribosomal head, base and platform (Lake, 1985) are all present in the hybrid 20S subunits, but both the head and base are smaller than in reconstituted 30S subunits (Fig. 3C). In the "quasi-symmetric" projection of hybrid subunits, shown in the last two columns of Figure 3A, the head and base of the subunits are more similar in size than in the equivalent *E. coli* projections, shown in the last column of Figure 3C.

Ribosomes were also isolated from kinetoplastid mitochondria and the small subunits were compared with the hybrid reconstituted subunits. Sedimentation of broken kinetoplasts on sucrose gradients (Fig 4) yielded several fractions in which 9S sequences were enriched. When fractions across the gradient were collected, run on a polyacrylamide gel and the gel probed for the 9S sequence in a Northern hybridization, 9S was found to be present mainly in fractions near the 40S peak illustrated in Figure 4 (data not shown). Additional experiments with both 9S and 12S probes indicate the presence of both RNA species in the 40S region (data not shown). These fractions were analyzed by electron microscopy and a representative field is shown in Figure 2. This fraction contains particles tentatively identified by the presence of classical ribosomal structures

Figure 4. Sucrose gradient sedimentation analysis of partially purified *L. tarentolae* small subunits. On the right side of the figure is the OD profile of mitochondrial ribosome subunits from *L. tarentolae* run on a 15-30% sucrose gradient (the break in the gradient is a change of scale). The hatched peak marked "40S" was collected for electron microscopy (Fig. 2). On the left is a methylmercury agarose gel analysis of the RNA content of the mitochondria preparation used for the gradient. RNA was visualized by Ethidium Bromide/UV. The positions of the 12S and 9S rRNAs are indicated. Note the relative absence of contamination with cytoplasmic rRNAs and their smaller fragments (Campbell *et al.*, 1987) (tRNAs were run off the gel).

as monomeric ribosomes (M), large (L) and small ribosomal subunits (marked by arrows) and, rarely (see the RNA gel in Fig. 4), cytoplasmic small ribosomal subunits.

Small subunits from this fraction are shown in Figure 3B and can be identified by the presence of the platform, head and base in the asymmetric projections of the subunit (frames 1, 2, 4 and 5, from the left, of Fig. 3B) and by the head and base in the quasi-symmetric projection of the subunit (frame 7 of Fig. 3B). Additionally, large subunits can be identified by the presence of the central protuberance and the L7/12 stalk (Lake, 1985) (see the two large subunits at the top-center of Fig. 2). The presence of ribosomes and their subunits is in accord with the results of the Northern hybridization that detect both 9S and 12S RNAs. [We assume that the large and small subunits have resulted from the dissociation of monosomes during preparation for microscopy.] The cytoplasmic small subunits are useful size references to compare with mitochondrial subunits, and a contaminating cytoplasmic subunit (enclosed by brackets) included in this field illustrates the small size of the mitochondrial subunits.

The close similarity of the small ribosomal subunits derived from mitochondria and from hybrid reconstitutions corroborates the identity of both. Individual mitochondrial small subunits are shown in Figure 3B for comparison with the hybrid reconstituted subunits (Fig. 3A). Key features present in both the mitochondrial particles and in the reconstituted hybrid particles, but differing from the features of reconstituted *E. coli* subunits (Fig. 3C) are (i) the tapered base, and (ii) the reduced head. Both types of particle are more like each other than either is like the *E. coli* small subunit. While these two particles closely resemble each other, they are not identical. This is reasonable since one contains ribosomal proteins from *E. coli* and the others are mitochondrial in origin.

In summary, successful heterologous reconstitution of a small ribosomal subunit using 9S mitochondrial RNA from a kinetoplastid protozoan has established that 9S forms small ribosomal subunits *in vivo* and provides evidence for the presence of a functional translation apparatus in the unusual mitochondrion of these cells.

ACKNOWLEDGEMENTS

We thank Angela Kost for expert electron microscopy, Jerome Arrington, Jr. for photography, and Maria Rivera and Tom Atha for helpful advice. Supported by grants from the NSF and NIH to J.A.L. and from the NIH to L.S.

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