Trypanosoma brucei mitochondrial ribonucleoprotein complexes which contain 12S and 9S ribosomal RNAs

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SUMMARY

Antibiotics have been widely used to identify ribosomal activity in *Trypanosoma brucei* mitochondria. The validity of some of the results has been questioned because the permeability of the trypanosome cell membrane for some antibiotics was not adequately addressed. Here we describe translation inhibition experiments with digitonin-permeabilized trypanosomes to exclude diffusion barriers through the cell membrane. Using this system we were able to confirm, next to the eukaryotic and thus cycloheximide-sensitive translation system, the existence of a prokaryotic-type translational activity being cycloheximide resistant, chloramphenicol sensitive and streptomycin dependent. We interpret this observation analogous to what has been found for other eukarya as the independent protein synthesis activity of the mitochondrial organelle. We further examined the putative translational apparatus by using isokinetic density-gradient analysis of mitochondrial extracts. The 2 mitochondrially encoded rRNAs, the 9S and 12S rRNAs, were found to co-fractionate in a single RNP complex, approximately 80S in size. This complex disassembled at reduced $MgCl_2$ concentrations into 2 unusually small complexes of 17·5S, containing the 9S rRNA, and 20S containing the 12S rRNA. A preliminary stoichiometry determination suggested a multicopy assembly of these putative subunits in a 2:3 ratio (20S:17·5S).

Key words: *Trypanosoma brucei*, mitochondrial translation, ribosomal RNA (rRNA), mitochondrial ribosomes.

INTRODUCTION

The unicellular haemoflagellates of the order Kinetoplastida, such as *Trypanosoma* and *Leishmania*, have been studied at large for their disease-causing attributes (Vickerman, 1985; Warren, 1988). However, in addition to the medical implications, kinetoplastid organisms have recently been used as model organisms to investigate novel RNA processing phenomena, such as *trans*-splicing (Ullu, Tschudi & Günzl, 1996) and RNA editing, a specific mitochondrial transcript maturation process (for recent reviews see Sloof & Benne, 1997; Kable, Heidmann & Stuart, 1997). Due to our interest in the RNA editing reaction, we re-examined mitochondrial protein biosynthesis in *Trypanosoma brucei* in order to address how edited mRNAs might be translated within the mitochondrial organelle.

Unfortunately, mitochondrial translation in kinetoplastid organism is poorly understood. The organisms have a single large mitochondrion with their mitochondrial DNA assembled in the form of a concatenated network, termed the kinetoplast (Englund *et al*. 1996). It consists of 2 types of circular DNA molecules, so-called mini and maxicircles, the latter being the equivalent of the conventional mitochondrial DNA. The maxicircle encoded rRNAs display only the barest resemblance to rRNAs in other organisms. They are extremely short, the small subunit rRNA is *ca*. 600 nucleotides in length with a Svedberg value of 9S, and the large subunit rRNA is a 12S molecule with *ca*. 1200 nucleotides. The 2 rRNAs can be folded into secondary structures with similarities to functionally important domains of the consensus structures of SSU and LSU rRNAs (Eperon *et al*. 1983; de la Cruz *et al*. 1985*a*, *b*; Sloof *et al*. 1985). However, the available data on the mitochondrial translation machinery are controversial. This uncertainty is routed mainly in 2 facts. First, antibiotic inhibition studies which aimed to distinguish between the cytosolic and mitochondrial translation event have been questioned because the permeability of the antibiotics into the trypanosomatid cells was not addressed in these experiments (Laub-Kupersztejn & Thirion, 1974; Kleisen & Borst, 1975; Spithill, Shimer & Hill, 1981). Second, the collected data on the structure and size of the mitochondrial ribosomes are inconclusive (Laub-Kupersztejn & Thirion, 1974; Hanas, Linden & Stuart, 1975; Scheinmann *et al*. 1993; Maarouf *et al*. 1995; Tittawella, 1995). Taken together, this tempted us to re-examine the translational activity and potential translational apparatus in *T. brucei* mitochondria using permeabilized trypanosome cells.

MATERIALS AND METHODS

Biochemicals

All chemicals were reagent grade or better. Cyclo-

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heximide stock solution was 2·5 mg/ml in 10 mM Tris–HCl, pH 8, 1 mM Na_2EDTA; chloramphenicol was 85 mg/ml in absolute ethanol; streptomycin was 50 mg/ml in double-distilled H_2O. Enzymes were obtained from either Boehringer Mannheim, Pharmacia or Stratagene and were used in accordance with the vendors' recommendations. Radioactive compounds were purchased from Amersham Buchler. Oligodesoxynucleotides were synthesized automatically using β-cyanoethyl phosphoramidite chemistry.

Cell growth and mitochondrial vesicle isolation

E. coli, strain HB101, was grown in LB broth to OD approximately 1·5 before harvesting for ribosome isolation. Procyclic *Trypanosoma brucei*, clone IsTaR1 (Stuart *et al*. 1984), was grown as described (Brun & Schönenberger, 1979). Cells were harvested at a cell density of 10^7 cells/ml and mechanically disrupted in a hypotonic buffer (1 mM Tris–HCl, pH 8, 1 mM Na_2EDTA). Mitochondrial vesicles were isolated by isopycnic centrifugation on preformed linear 15–35 % (v/v) Percoll gradients (Harris, Moore & Hajduk, 1990).

Amino acid incorporation assay

Trypanosome cells were incubated with digitonin at a concentration of 25 μg/mg protein in 50 mM Tris–HCl, pH 7·5, 0·1 mM Na_2EDTA, 0·7 M sorbitol, 0·1 % (w/v) bovine serum albumin (BSA) at room temperature for 15 min. After several washes, the cells were resuspended in the same buffer without BSA. Aliquots of treated cells were incubated with the antibiotics at final concentrations as specified in each experiment and with 1·5 μM [^{35}S]methionine (specific activity 1000 Ci/mmol) at 25 °C for 30 min. After incubation, the methionine concentration was adjusted to 10 mM with non-radioactive methionine and 10 μl of the reaction was incubated with 1 ml of 5 % (w/v) trichloroacetic acid (TCA) at room temperature for 30 min. After heating at 70 °C for 15 min, the precipitates were collected on nitrocellulose membranes (Millipore, HA) pre-treated with 100 mM methionine. Filters were counted in scintillation fluid.

Ribosome isolation

The ribosome isolation from *E. coli* followed the protocol of Spedding (1990). Mitochondrial RNP complexes were isolated from mitochondrial vesicles by lysis in 6 mM HEPES, pH 7·5, 100 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0·2 % (v/v) NP-40. After centrifugation at 15600 ***g*** for 10 min the cleared supernatant was layered on top of an equal volume of 1·1 M sucrose in 50 mM Tris–HCl, pH 7·5, 100, 250 or 500 mM NH_4Cl, 10·5 mM $MgCl_2$, 0·5 mM Na_2EDTA, 3 mM DTT. After centrifugation at 100000 ***g*** for 15 h at 4 °C, the crude ribosomal pellet was resuspended in 10 mM Tris–HCl, pH 7·5, 60 mM NH_4Cl, 10 mM $MgCl_2$, 3 mM DTT. Insoluble material was pelleted by centrifugation at 15600 ***g*** for 1 min, and the supernatant was analysed on isokinetic density gradients (see below). After centrifugation and fractionation, the 80S and 60–65S regions were pooled and diluted with an equal volume of the above-mentioned ribosome suspension buffer. The particles were recovered by centrifugation at 56000 ***g*** for 24 h at 4 °C.

Isokinetic gradient analysis

Throughout this study, sedimentation analysis was performed on linear 5–30 % (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7·5, 50 mM NH_4Cl, 0·1, 0·5, 5, or 10 mM $MgCl_2$ as specified, and 3 mM DTT. Samples were fractionated at 52000 ***g*** for 16 h at 4 °C. Apparent Svedberg values were estimated by comparison with *E. coli* ribosomes (70S), ribosomal subunits (50S and 30S), ribosomal RNAs (23S and 16S), and yeast $tRNA^{Phe}$ (4S) as markers.

Subunit stoichiometry determination

An estimate of the ribosomal subunit stoichiometry was gained by determining the ratio of SSU rRNA (9S) molecules versus LSU rRNAs (12S) within the 80S complexes. RNA from 80S RNP peak fractions was isolated by deproteinization with phenol and ethanol precipitation. Serial dilutions of the material (in triplicate) were dot-blotted onto nylon membranes and probed with 5′-end labelled oligodeoxynucleotides complementary to the 12S and 9S rRNAs. Hybridization signals were quantitated by densitometry, and absolute amounts were calculated in comparison with 12S and 9S rRNA standard curves prepared from known quantities of serially diluted mitochondrial RNA preparations and probed as above along with the 80S RNP samples.

Dot blot and Northern hybridization analysis

RNA species were monitored by dot-blot or Northern hybridization. Radio-isotope labelled oligodeoxynucleotides were used as probes following conditions as described previously (Shu *et al*. 1991). The following oligodeoxynucleotides were used: 9S rRNA: 5′-CCGCAACGGCTGGCATCC-3′; 12S rRNA: 5′-GGAGAGTAGGACTTGCCCTA-3′. Autoradiograms were analysed by densitometry.

RESULTS

Is there a translational activity in trypanosome mitochondria?

Due to the above-mentioned controversy concerning the antibiotic sensitivity or antibiotic resistance of

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Table 1. Relative translation activity

Antibiotics	Permeabilized cells (%)	Intact cells (%)
−CHX*	100	100
+CHX	9	8
+CHX/+CAP (0·042 mg/ml)	7	
+CHX/+CAP (0·142 mg/ml)	2	
+CHX/+CAP (1·7 mg/ml)		10
+CHX/+SM		12

* CHX, cycloheximide; CAP, chloramphenicol; SM, streptomycin.

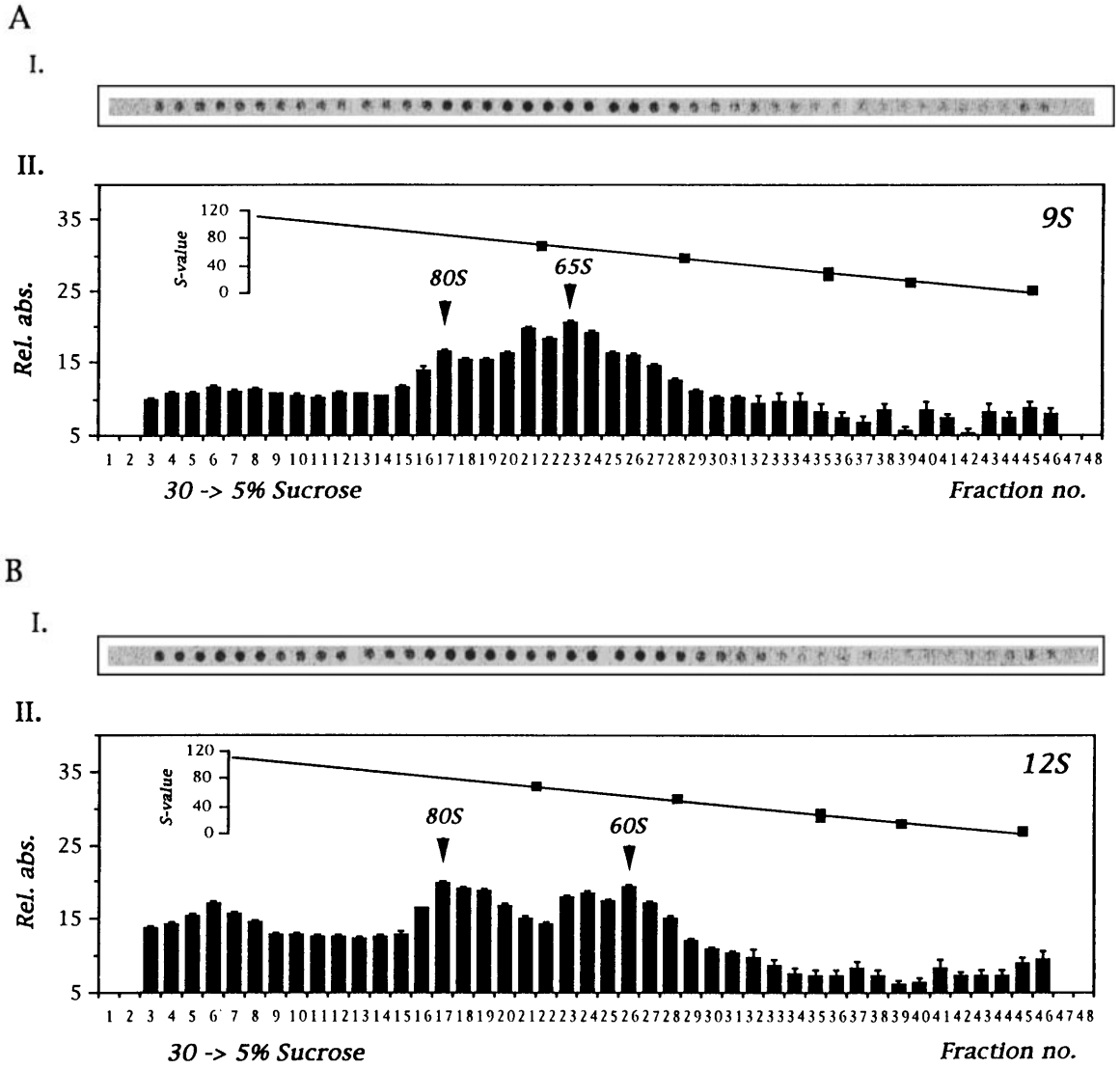

Fig. 1. Sedimentation analysis of *Trypanosoma brucei* mitochondrial extracts assaying for the distribution of the 9S (A) and 12S rRNAs (B). The dot-blot hybridization results are shown in Panel I and the relative intensities of the hybridization signals were quantitated by densitometry and plotted in relative absorbance units (Panel II). Error bars represent standard deviations. The apparent Svedberg values were determined as described in the Materials and Methods (insert in Panel II).

the translation activity in intact trypanosome cells (Laub-Kupersztejn & Thirion, 1974; Hanas *et al.* 1975; Kleisen & Borst, 1975; Spithill, Shimer & Hill, 1981), we re-examined the amino acid incorporation activity of trypanosomes in response to different antibiotics by using digitonin-permeabilized cells (Turren, 1989). Digitonin-treated trypanosomes were incubated with [^{35}S]methionine

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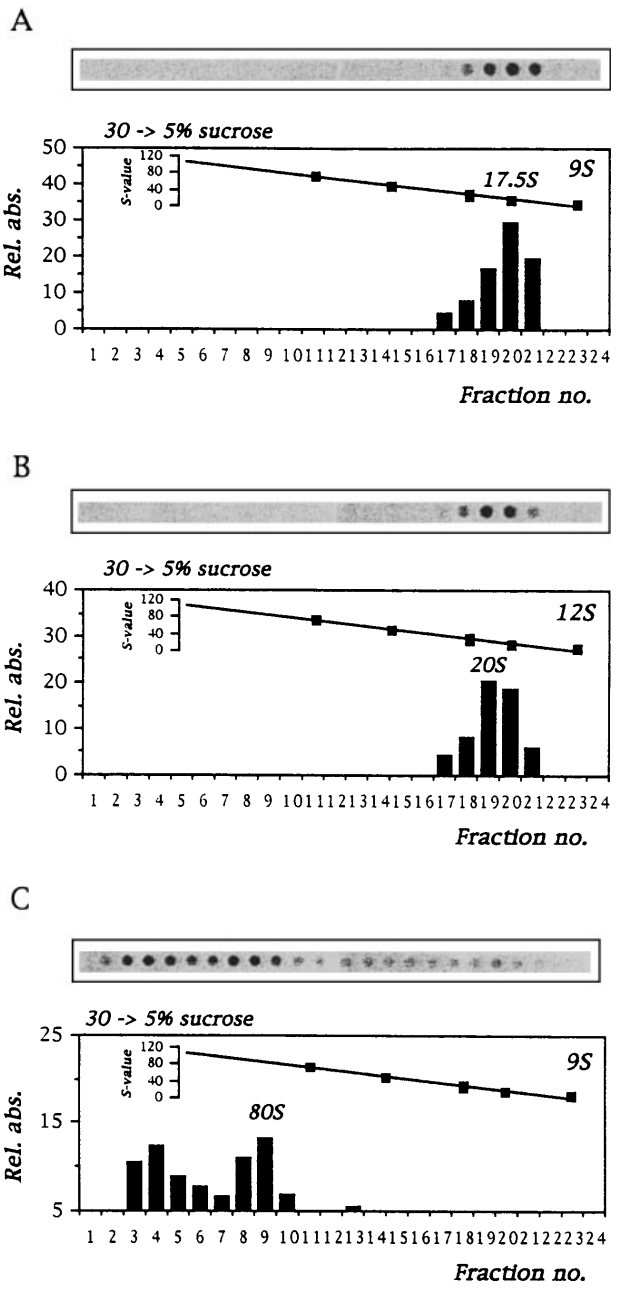

Fig. 2. Disassembly of the 80S complex at reduced $MgCl_2$ concentrations. The 80S complexes were dialysed at 5 mM $MgCl_2$, fractionated on a linear 5–30 % (w/v) sucrose gradient and the distribution of the 9S (A) and 12S rRNAs (B) across the gradient was measured. (C) Non-dialysed control 80S RNP sample at 10 mM $MgCl_2$ that was treated identically throughout the experiment. Fractions were probed for the presence of the 9S rRNA. Annotations are as in the legend to Fig. 1.

in the presence or absence of various antibiotics. Incorporation of the radioactive amino acid into precipitable material was measured by scintillation counting after trichloroacetic acid (TCA) precipitation.

As shown in Table 1, we were able to confirm the previous observation that the whole cell amino acid incorporation activity was sensitive to the eukaryotic-type translational inhibitor cycloheximide (Laub-Kupersztejn & Thirion, 1974; Hanas *et al.* 1975; Kleisen & Borst, 1975; Spithill *et al.* 1981). Ninety-one percent of the incorporation activity could be blocked by 50 μg/ml cycloheximide which we interpret as the inhibition of the cytosolic translational apparatus. Nine percent of the [^{35}S] methionine incorporation remained unaffected by cycloheximide but was sensitive to chloramphenicol, a bacterial-type translational inhibitor. At a concentration of 42 μg/ml chloramphenicol this activity was reduced to 7 % and increasing the chloramphenicol concentration to 142 μg/ml resulted in only 2 % of the [^{35}S]methionine incorporation activity remaining (Table 1). Thus, chloramphenicol was able to reduce the cycloheximide-resistant translational activity in a concentration-dependent manner and identical results were obtained using isolated *T. brucei* mitochondrial vesicles (data not shown).

In addition to chloramphenicol we tested the effect of streptomycin, another bacterial-type translational inhibitor. Interestingly, in the presence of streptomycin we measured an increase in the [^{35}S]-methionine incorporation activity up to a maximum of 135 % at a concentration of 2·1 mg/ml streptomycin/mg protein (data not shown). Such a phenomenon has been described before for certain *E. coli* mutants and was interpreted as a stabilization effect of the rRNA by the antibiotic thus restoring translational activity (Hummel, Ahmad & Böck, 1983).

Lastly, we asked if we could reproduce these results with intact trypanosomes to address the question of whether the various antibiotics can permeate the cell membrane. As shown in Table 1, we measured 92 % inhibition of [^{35}S]methionine incorporation due to the presence of cycloheximide, identical to what we had found for digitonin-permeabilized cells. The cycloheximide-resistant activity could be increased by the addition of streptomycin (up to 140 %) but was not sensitive to chloramphenicol even at a concentration of 1·7 mg/ml. These results clearly indicated that cycloheximide and streptomycin are able to permeate the *T. brucei* cell membrane, but chloramphenicol, as suggested before (Kleisen & Borst, 1975; Spithill *et al.* 1981), is not.

In summary, the existence of approximately 10 % cycloheximide-resistant but chloramphenicol-sensitive and streptomycin-dependent protein synthesis argues for a second 'non-eukaryotic'-type translational activity in *T. brucei*, which we suggest is due to mitochondrial protein synthesis.

Are there ribosomal particles?

Based on the data described above, we took a further step to examine the putative translational apparatus in *T. brucei* mitochondria. The mitochondrial genome of kinetoplastid organisms encodes 2 ribo-

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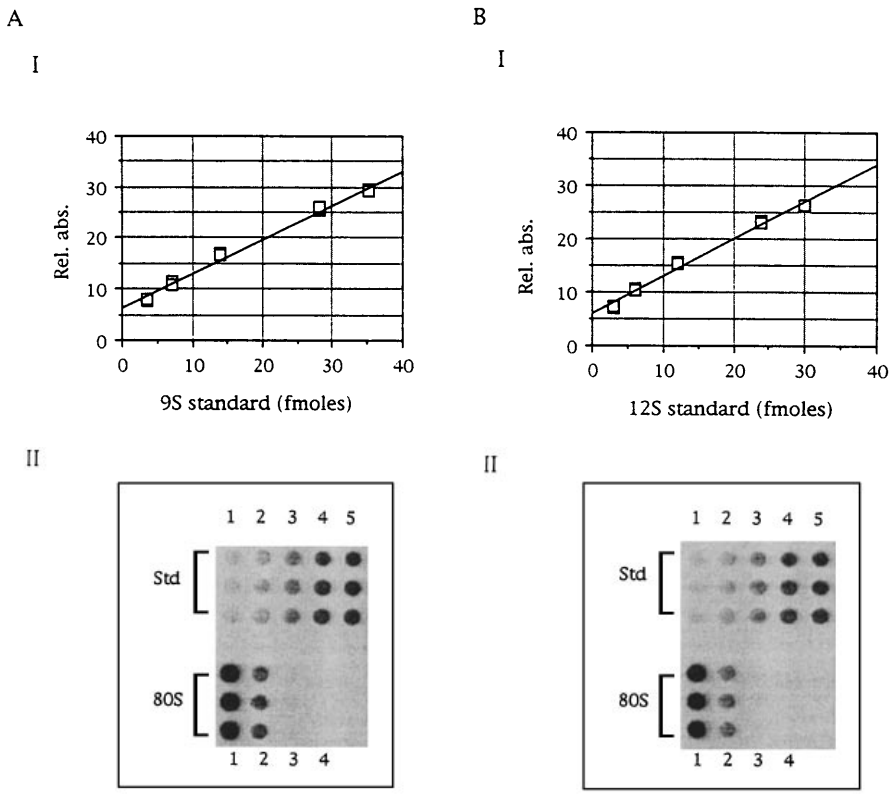

Fig. 3. Estimation of the subunit stoichiometry of the 80S complex using the 9S rRNA and the 12S rRNA as subunit markers. The ratio of 9S (panel A) versus 12S rRNA (panel B) was determined from dot-blot probing experiments of serial dilutions (1–5) of RNA material extracted from 80S RNP complexes (II). Absolute quantities were calculated by comparison to standard curves (I) constructed from known quantities of 9S and 12S rRNA (Std). The curves were fitted by a linear least square regression analysis of triplicate samples with correlation coefficients of 0·992 (9S), 0·990 (12S).

somal RNAs, a 9S rRNA molecule resembling SSU rRNAs and a 12S homologue of the LSU rRNA. If these molecules are functional ribosomal RNAs they should assemble with ribosomal proteins to form high molecular weight ribosomal RNP complexes (Scheinman *et al*. 1993; Tittawella, 1995) and these complexes should be present in mitochondrial extracts of *T. brucei*. For this reason we performed a sedimentation analysis of mitochondrial extracts on isokinetic density gradients to identify potential high molecular weight ribonucleoprotein complexes containing both rRNA molecules. At 50 mM monovalent (NH_4^+) and 10 mM divalent (Mg^{2+}) cation concentration the 9S rRNA fractionated as part of 2 broad peaks equivalent to complexes of approximately 80S and 65S apparent size (Fig. 1A). Equally, the 12S rRNA was identified within 2 complexes of the approximate sizes of 80S and 60S (Fig. 1B). No free rRNA material could be detected, but very high molecular weight complexes ($\geqslant$ 120S) could be seen. Pre-treatment of the ribosome preparation with proteinase K resulted in sedimentation of both rRNAs with apparent S-values identical to free rRNA molecules (data not shown), confirming the association of the rRNAs with proteins.

With the conventional 2 subunit ribosomal structure in mind, we further examined the 80S complex, where the 12S and 9S rRNAs co-fractionated. If the 80S complex is a fully assembled ribosome, it should dissociate at reduced Mg^{2+} concentrations (Chao, 1957; Tissières & Watson, 1958). The 80S complexes were collected from a 5–30% (w/v) sucrose gradient, and dialysed against buffer containing 5 mM $MgCl_2$ at 4 °C. After separation on a 5–30% (w/v) sucrose gradient containing 5 mM $MgCl_2$, the fractionation of the 9S and 12S rRNAs was determined as above (Fig. 2A, B). The result showed that the 80S complex disassembled: a 12S rRNA-containing complex with an apparent Svedberg value of 20S and a 9S rRNA-containing complex of 17·5S were detected. The untreated sample clearly did not disassemble (Fig. 2C) and, instead, partial aggregation was observed.

What are the 60S and 65S complexes?

The dissociation behaviour of the 80S complex at reduced $MgCl_2$ concentration was inconsistent with the hypothesis that the 65S and 60S complexes were subunits of the 80S RNP. In order to get a first insight as to the function of the 65S and 60S complexes, the biochemical properties of these 2 complexes were examined. The 60S and 65S RNP complexes were apparently stable at reduced $MgCl_2$ con-

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centrations as indicated by the presence of the 60S and 65S complexes after dialysis of the crude ribosome preparations against a buffer containing no Mg^{2+}. Under the same conditions the 80S complexes disassembled as expected (data not shown). Further reducing the $MgCl_2$ concentration by adjusting the Na_2EDTA concentration of 0·9 mM, both complexes disassembled into molecules with apparent S-values identical to free rRNAs (data not shown). When the RNP preparations were digested with 5 μg/ml RNase A, only the 65S and the 60S complexes remained unaffected (data not shown).

Is the 80S complex assembled of multiple subunits of the 20S and 17·5S RNPs?

In order to identify other RNA molecules as potential components of the 80S RNP complex we deproteinized gradient-purified 80S particles and radio-isotope labelled the RNA material at their 5′-ends with and without prior dephosphorylation. Only 3 RNA species were labelled: the 2 mitochondrial ribosomal RNAs (12S and 9S) and molecules 60–70 nucleotides in length, very probably representing ribosome bound tRNAs (data not shown). Thus, no other RNA species could be identified, which prompted us to ask if the 80S RNP could potentially be assembled of multiple subunits of the 12S rRNA-containing 20S complex and the 9S rRNA-containing 17·5S RNP. Therefore, we determined the stoichiometry of the 2 rRNAs within the 80S complex by dot-blot hybridization and the results are shown in Fig. 3. By comparison with standard curves we calculated a 12S:9S rRNA ratio of 16 fmol:23 fmol, equivalent to a relative ratio of 1:1·5 or 2:3.

DISCUSSION

The existence of mitochondrial protein synthesis in kinetoplastid organisms is a question that has not been directly demonstrated. The present study describes translation inhibition experiments using intact trypanosomes, as well as digitonin-treated cells. Digitonin treatment was used to exclude permeability barriers, which had been suggested to have caused contradictory results. Our data clearly demonstrated that 90% of the translational activity exhibited features typical of eukaryotic protein synthesis and that chloramphenicol was not able to penetrate the intact trypanosome cell membrane. Ten percent of the total translational activity, however, was sensitive to chloramphenicol in permeabilized cells and indicated a second, prokaryotic-type translational system present. By analogy to other eukaryotes we attribute this activity to a mitochondrially located protein synthesis machinery.

In addition to addressing the effects of chloramphenicol on translation we also monitored the effect of streptomycin. Streptomycin has been shown to bind to the SSU rRNA thereby causing the ribosome to malfunction. Streptomycin-resistant mutants in *E. coli* have changes in either the SSU rRNA or in ribosomal protein S12 (Cundliffe, 1990), and an S12 homologue (RPS12) has been identified on the maxicircle DNA in trypanosomatid organisms (Maslov *et al*. 1992). Interestingly, streptomycin enhanced the cycloheximide-resistant translational activity, identical to that found for streptomycin-dependent mutants in *E. coli*. These mutants are characterized by their ribosome assembly defects which can be compensated for by the binding of the antibiotic which restores translational activity (Hummel *et al*. 1983). A similar enhancement effect has been shown in *T. brucei* for erythromycin which binds to the LSU rRNA (Spithill *et al*. 1981). In essence, these results argue for a quasi 'labile' mitochondrial ribosome structure in *T. brucei* which can be stabilized by the binding of aminoglycoside antibiotics.

We were able to identify several high molecular weight RNP complexes containing the 9S and 12S rRNA molecules in *T. brucei* mitochondrial extracts. The 9S rRNA was assembled in 2 complexes, 80S and 65S in approximate size and the 12S rRNA was complexed in *ca*. 80S and 60S RNPs. Since the 80S particle assembled both rRNA molecules, it is tempting to interpret it as the putative mitochondrial ribosome. However, this complex dissociated at reduced Mg^{2+} concentrations into 2 unusually small RNPs: a 9S rRNA-containing complex with an apparent S-value of 17·5S and the 12S rRNA-containing RNP of 20S. The value of 17·5S for the 9S rRNA-containing RNP is in good agreement with data from a more recent report by Scheinman *et al*. (1993). These authors were able to assemble *Leishmania tarentolae* 9S rRNA with the *E. coli* complement of small subunit ribosomal proteins, which resulted in a 20S RNP complex. Nevertheless, the 17·5S and 20S particles cannot assemble in the conventional 1:1 ribosomal subunit stoichiometry to form an 80S complex and thus other explanations have to be envisaged. Among several possibilities we favour 2 scenarios. In one, the putative subunits might be unstable at reduced magnesium concentration leading to the dissociation of a significant number of subunit components. The 9S and 12S rRNAs could assemble with an extremely large number of proteins ultimately to form the 80S RNP. However, animal mitochondrial ribosomes with rRNA molecules of 16S and 12S are assembled with 85 proteins and only amount to a Svedberg value of 55S (Matthews *et al*. 1982). Thus, even more proteins would be required to bind to the 12S and 9S rRNAs. An alternative is that additional RNA molecules are involved. Since precedent for a

non-covalent assembly of fragmented ribosomal RNAs into mitochondrial ribosomes exists (Gray & Boer, 1988), we searched for additional RNA components, with an essentially negative result, however.

Secondly, the subunit stoichiometry of the 80S RNP might be different from the conventional ribosomal 1:1 ratio. Our determination of the relative proportion of 12S rRNA versus 9S rRNA molecules within the 80S complex revealed a 2:3 ratio. Considering the determined apparent S-values of the 12S and 9S rRNA-containing complexes of 20S and 17·5S, this would amount to an additive value of 92·5S. However, Svedberg values are not additive, and fully assembled particles have reduced S-values when compared to the sum of the subunits due to compactation upon subunit association. Therefore, the additive value of 92·5S is in good agreement with the value of 80S for the fully assembled RNP. Although we feel that additional experimental evidence is required to substantiate our observation, the non-stoichiometric assembly of the 80S complex from the putative subunits is especially attractive from the point of view that it provides a simple explanation for (1) the obvious lack of rRNA material within the putative ribosome and (2) the observed stimulation of the translational activity by streptomycin.

The existence of the 60S and 65S complexes was unexpected. These complexes were stable at reduced Mg^{2+} concentrations, and were resistant to nuclease treatment under our conditions. They are unlikely subunits of the 80S RNP since 2 particles with apparent Svedberg values of 60S and 65S would require a tremendous structural refolding to assemble into an 80S complex. Because of their stable nature, they might be storage particles for the 2 rRNAs. The presence of a 60S mitochondrial RNP particle has been reported for another kinetoplastid organisms, *Crithidia luciliae*, but this complex dissociated into subunits of 44S and 32S (Laub-Kupersztejn & Thirion, 1974) thus showing different properties from the 60S complex described above. Similarly, the characteristics of the 72S RNP identified in *T. brucei* (Hanas *et al*. 1975) and the 75S RNPs reported in *L. donovani* and *L. tropica* (Maarouf *et al*. 1995) did not parallel the features of the 80S or 60S and 65S complexes described here.

In summary, our translation inhibition experiments with permeabilized cells confirmed the existence of a mitochondrial type translational activity in *T. brucei*. The activity could be stimulated by streptomycin, suggesting a stabilization of the translational apparatus by the binding of aminoglycoside antibiotics. A candidate ribosomal RNP complex of 80S was identified containing the 2 mitochondrially encoded ribosomal RNA molecules but displaying either a non-typical ribosomal subunit structure or unusual subunit stability features.

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