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SHORT COMMUNICATION

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Chloramphenicol-sensitive mitochondrial translation in *Trypanosoma brucei*

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Abstract We developed an *in organello* system to label newly synthesized mitochondrially encoded proteins of *Trypanosoma brucei*. Highly purified mitochondria, prepared under isotonic conditions, were incubated with radioactive methionine and cysteine in a suitable translation buffer. Analysis of mitochondrial extracts on TRIS-Tricine gels revealed a subset of labeled, NP-40-insoluble proteins. The labeling of these proteins was resistant to the cytosol-specific translation inhibitor cycloheximide. The proteins, however, were not labeled in the presence of chloramphenicol or erythromycin, inhibitors of prokaryotic type translation, or puromycin, a general translation inhibitor. These results indicate that isotonically isolated mitochondria of *T. brucei* are capable of protein synthesis.

Introduction

Mitochondrial translation in trypanosomatids is of great interest for at least three reasons. First, many RNAs that are synthesized in trypanosomatid mitochondria undergo RNA editing by uridylate insertions and deletions to become functional mRNAs. In recent years, much has been learned concerning the mechanism of RNA editing (Sollner-Webb 1996). However, nothing is known about one important aspect of the problem, whether and, if so, how fully edited mRNAs are translated within mitochondria. Second, mitochondrial ribosomes of trypanosomatids contain the shortest known rRNAs among all eukaryotes and may therefore serve as a system for investigation of the minimal requirements for a protein synthesis machinery (Benne and Sloof 1987). Third, unlike mitochondrial translation in most other species, mitochondrial protein synthesis in trypanosomatids relies exclusively on tRNAs that are imported from the cytosol (Schneider 1994).

The problem of mitochondrial translation in trypanosomatids has proved to be difficult to analyze. Attempts to purify mitochondrial ribosomes have been only partially successful (Shu and Göringer 1998). Cytochrome c oxidase from *Crithidia fasciculata* has been purified. However, all the putative mitochondrially encoded subunits were refractory to protein sequencing (Speijer et al. 1996; Brekk et al. 1997). The best evidence for mitochondrial translation was found in a number of studies using antibodies raised against synthetic peptides of putative mitochondrially encoded proteins that detected signals of the expected molecular weights in the mitochondrial fraction (Shaw et al. 1989; Beattie and Howton 1996). In addition, *in organello* labeling experiments in *C. fasciculata* identified a number of putative mitochondrially encoded proteins. However, no information was provided as to whether the observed translation was sensitive to inhibition by prokaryotic type translation inhibitors (Tittawella 1998). In the present study we used an *in organello* labeling approach for direct demonstration of mitochondrial translation in *Trypanosoma brucei*.

Material and methods

Cells

Procyclic wild-type and transformed *Trypanosoma brucei*, stock 427, were grown in SDM-79 medium supplemented with 5% fetal bovine serum.

In organello translation

Mitochondria were isolated using sterilized buffers and equipment as described elsewhere (Hauser et al. 1996), except that a low-speed

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spin (300 *g*) was routinely performed before loading of the Nycodenz gradients. The absence of intact *T. brucei* cells and bacterial contamination in isolated mitochondrial preparations was confirmed by light microscopy. *In organello* translations were performed using 400 μg of isolated mitochondria each in 100 μl of translation buffer [20 m*M* TRIS-HCl (pH 7.4), 30 m*M* KH_2PO_4, 0.6 *M* sorbitol, 5 m*M* succinate, 50 m*M* KCl, 20 m*M* $MgSO_4$, 12 m*M* creatine phosphate, creatine phosphokinase at 0.16 mg/ml, 4 m*M* ATP, 0.5 m*M* GTP, 5 m*M* NADH, fatty-acid-free bovine serum albumin at 2.5 mg/ml, and 1 m*M* of all biological amino acids except methionine and cysteine] containing 1 μ*M* (approximately 150 μCi) of a mixture of ^{35}S-labeled methionine and cysteine (Pro-mix, Amersham, Switzerland). The reactions were incubated for 90 min at 25 °C, and mitochondria were reisolated by centrifugation for 5 min at 5,200 *g* at 4 °C. The resulting pellets were extracted with 100 μl of 50 m*M* potassium phosphate (pH 7.5) containing 0.5% NP-40. Subsequently, the samples were centrifuged at 15,000 *g* for 15 min, and the pellet was solubilized under constant mixing for 2 h at 37 °C in 50 μl of 0.28 *M* TRIS-HCl (pH 6.8), 2.8% sodium dodecyl sulfate, 5% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. All reactions were analyzed on TRIS-Tricine gels (Schägger and von Jagow 1987), which were first stained with Coomassie brilliant blue to check for equal loading and then processed for fluorography.

In organello translation reactions were incubated in the presence or absence of cycloheximide at 100 μg/ml, at water-soluble chloramphenicol 50 μg/ml (Sigma, Switzerland), erythromycin at 100 μg/ml, 1 m*M* puromycin, or a mixture of 2 μ*M* valinomycin and 50 μ*M* carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP).

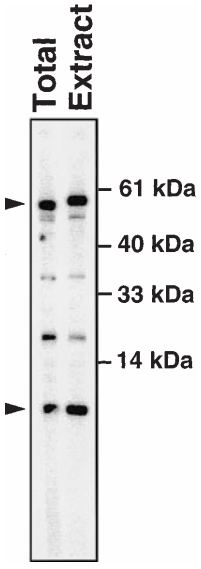

Fig. 1 NP-40 extraction of mitochondria leads to enrichment of the ATPase complex. In all, 20 μg of total mitochondrial protein and 10 μg of the 0.5% NP-40 pellet were analyzed with an antiserum directed against the ATPase complex of *C. fasciculata* and iodinated protein A. The antiserum recognized two components (*arrows*) of the ATPase complex in *T. brucei*, both of which showed 3- to 5-fold enrichment in the NP-40 pellet

Results and discussion

Mitochondria isolated by conventional hypotonic purification procedures (Braly et al. 1974; Harris et al. 1990) are not capable of synthesizing proteins in an *in organello* system (data not shown). We therefore used mitochondria isolated under isotonic conditions using nitrogen cavitation followed by Nycodenz gradients. Mitochondria isolated by this method had previously been shown to exhibit a membrane potential and to be capable of importing mitochondrial precursor proteins (Hauser et al. 1996). Mitochondria were incubated under suitable buffer conditions with radioactive methionine and cysteine for 60 min at 27 °C. After incubation, mitochondria were extracted with 0.5% NP-40 and the labeled proteins were analyzed on TRIS-Tricine gels optimized to separate hydrophobic proteins (Schägger and von Jagow 1987). The respiratory complexes, which include practically all mitochondrially encoded proteins, are relatively insoluble in 0.5% NP-40 and should therefore be enriched in the pellet (Speijer et al. 1996).

The immunoblot depicted in Fig. 1 shows that the ATPase complex is indeed enriched in the 0.5% NP-40 pellet by a factor of 3–5. During the *in organello* assay, radioactive methionine and cysteine is incorporated into a subset of distinct proteins found in the NP-40 pellet. Labeling of the proteins is not altered in the presence of cycloheximide, an inhibitor of translation in the eukaryotic cytosol. This indicates that the observed labeling pattern was not due to cytosolic contamination or to a small residual number of intact cells that might have remained in the mitochondrial fraction. However, if puromycin, a tRNA analogue that blocks prokaryotic as well as eukaryotic translation, is added, no labeled protein is found.

Most importantly, incorporation of radioactivity into respiratory complexes was sensitive to erythromycin and chloramphenicol, which have been shown in other systems to inhibit mitochondrial and prokaryotic translation only. Antibiotic inhibition studies are the most powerful tools for the operational definition of mitochondrial translation. However, previous studies have led to contradictory results and have been questioned because the permeability of the trypanosomatid cell membrane has not been addressed (Laub-Kupersztejn and Thirion 1974; Kleisen and Borst 1975; Spithill et al. 1981). These problems can be avoided by the use of permeabilized cells or, as in the present study, by the use of isolated organelles. Our results fully agree with the study of Shu and Göringer (1998) and extend it further, showing that radioactivity is indeed incorporated into proteins. In summary, the two studies firmly establish that mitochondrial translation in trypanosomes, as in all other eukaryotes, is sensitive to chloramphenicol.

Surprisingly, choramphenicol sensitivity has not been tested in a recent study on *in organello* translation in *C. fasciculata* (Tittawella 1998). A comparison of our data with these experiments is therefore problematic; nevertheless, an apparently similar labeling pattern was obtained. In our study, approximately 10–12 putative mitochondrially encoded proteins were detected. The apparent molecular weights of the main erythromycin- and chloramphenicol-sensitive labeled proteins (asterisks in Figs. 1, 2) correspond to 17.8, 22.9, and 27 kDa,

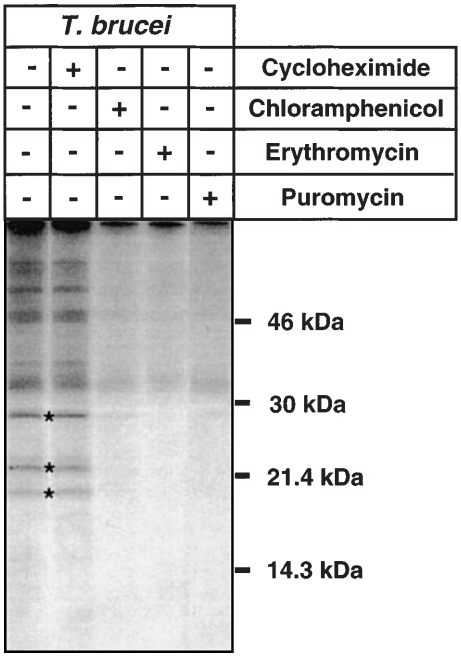

Fig. 2 Mitochondrial translation in *T. brucei* is sensitive to prokaryotic type translation inhibitors. NP-40 pellets of *in organello* labeling reactions performed in the absence or presence of cycloheximide, chloramphenicol, erythromycin, and puromycin were analyzed on TRIS-Tricine gel. The positions of molecular-weight markers are indicated *on the right*. The three main translation products in mitochondria are indicated by *asterisks*

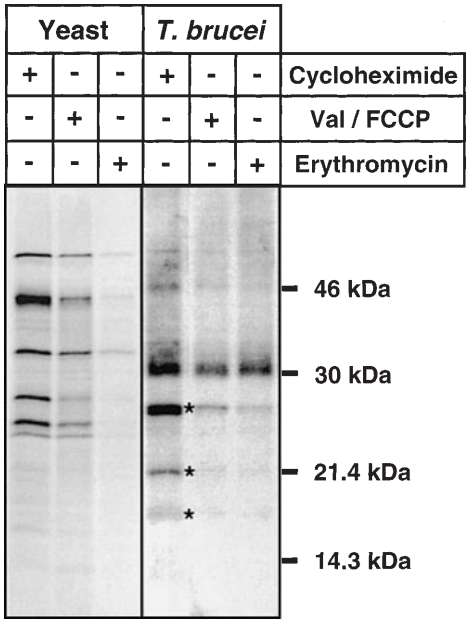

Fig. 3 Mitochondrial translation in *S. cerevisiae* and *T. brucei* are comparable. *Left panel*: 20 µg of isolated yeast mitochondria was subjected to *in organello* labeling in the presence of cycloheximide, a mixture of valinomycin and FCCP, or erythromycin and then analyzed on the same TRIS-Tricine gel. *Right panel*: same conditions described above except that 200 µg of isolated trypanosomal mitochondria was used in each labeling reaction and the samples were subjected to 0.5% NP-40 extraction prior to the analysis. The three main translation products of *T. brucei* mitochondria are indicated by *asterisks*

respectively, which lie within the range of several proteins predicted to be encoded in the mitochondrion of *T. brucei*. However, the present results do not allow their identification.

Finally, *in organello* translation in trypanosomes and in the yeast *Saccharomyces cerevisiae* was compared (Fig. 3). As expected, similar numbers of labeled proteins were found, which did not have exactly corresponding sizes in the two organisms. In both species, mitochondrial translation was sensitive to erythromycin. Finally, if the membrane potential was dissipated by valinomycin and FCCP, mitochondrial translation was reduced in the yeast and abolished in *T. brucei*. Transport of amino acids across the inner membrane requires a membrane potential. It is therefore likely that the reduction in translation observed in the absence of a membrane potential was an indirect effect due to reduced uptake of amino acids. Nevertheless, such an effect is expected to be specific for mitochondria.

In summary, our results present for the first time direct evidence for translation in isolated mitochondria of *T. brucei*. The observed mitochondrial protein synthesis shows the expected properties of being resistant to translation inhibitors of the cytosolic type but being sensitive to prokaryotic type translation inhibitors. Future work will focus on the identification of the mitochondrially synthesized gene products.

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