

Non-canonical Eukaryotic Glutaminyl- and Glutamyl-tRNA Synthetases Form Mitochondrial Aminoacyl-tRNA in *Trypanosoma brucei**

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Glutaminyl-tRNA synthetase is thought to be absent from organelles. Instead, Gln-tRNA is formed via the transamidation pathway, the other route to this essential compound in protein biosynthesis. However, it was previously shown that glutaminyl-tRNA synthetase activity is present in *Leishmania* mitochondria. This work identifies genes encoding glutaminyl- and glutamyl-tRNA synthetase in the closely related organism *Trypanosoma brucei*. Down-regulation of their respective gene products by RNA interference showed that (i) they are essential for the growth of insect stage *T. brucei* and (ii) they are responsible for essentially all of the glutaminyl- and glutamyl-tRNA synthetase activity detected in both the cytosol and the mitochondria. *In vitro* aminoacylation experiments with the recombinant *T. brucei* enzymes and total tRNA confirmed the identity of the two aminoacyl-tRNA synthetases. Interestingly, *T. brucei* uses the same eukaryotic-type glutaminyl-tRNA synthetase to form mitochondrial and cytosolic Gln-tRNA. The formation of Glu-tRNA in mitochondria and the cytoplasm is catalyzed by a single eukaryotic-type discriminating glutamyl-tRNA synthetase. *T. brucei*, similar to *Leishmania*, imports all of its mitochondrial tRNAs from the cytosol. The use of these two eukaryotic-type enzymes in mitochondria may therefore reflect an adaptation to the situation in which the cytosol and mitochondria use the same set of tRNAs.

For protein synthesis, most organisms require 20 functionally different aminoacyl-tRNA synthetases, one for each amino acid. These enzymes attach the cognate amino acid to the corresponding tRNA species, e.g. glutaminyl-tRNA synthetase (GlnRS)¹ acylates tRNA^{Gln} with glutamine. However, in many organisms, the formation of Gln-tRNA does not proceed

by direct aminoacylation. Rather, Gln-tRNA is synthesized in a tRNA-dependent amidation of glutamate mischarged to tRNA^{Gln}. Genomic and biochemical analyses indicate that the pathways of Gln-tRNA formation evolved as distinct systems in the three kingdoms (1). The eukaryotic cytoplasm uses GlnRS exclusively, whereas bacteria and archaea utilize the transamidation pathway. Because of the bacterial origin of organelles, the transamidation pathway is expected to be present in chloroplasts and mitochondria. Biochemical data have shown that most organelles lack GlnRS activity and instead use the indirect transamidation pathway to Gln-tRNA formation (2).

Whereas in many eukaryotes all of the tRNAs necessary for organellar translation are encoded on the mitochondrial genome, there are a large variety of organisms in which a variable number of apparently essential mitochondrial tRNA genes are missing. It has been shown that the lack of these genes is generally compensated by import of the corresponding cytosolic tRNAs (3). In all of the cases known, imported tRNAs originate from nuclear genes encoding eukaryotic-type tRNAs also utilized in cytosolic translation. An extreme situation is found in the trypanosomatids such as *Trypanosoma brucei* and *Leishmania*, which have lost the entire set of mitochondrial tRNA genes. These organisms must import all of the tRNAs necessary for organellar protein synthesis. As a result, the bacterial-type translation system of the mitochondria is forced to function with imported eukaryotic-type tRNAs only (4). Surprisingly, it was shown that a mitochondrial GlnRS activity exists in *Leishmania* (5). To investigate this finding at the genome level, we decided to characterize Gln-tRNA and Glu-tRNA formation in *T. brucei* in which RNA interference methodology (6) and biochemical analysis could be used.

EXPERIMENTAL PROCEDURES

Cells—Procytic *T. brucei*, stock 427, was grown at 27 °C in semi-defined medium-79 medium supplemented with 5% fetal calf serum. Cells were harvested at 3.5–4.5 × 10⁷ cells/ml. Procytic *T. brucei*, strain 29–13, on which the RNAi knock-down cell lines are based was grown in semi-defined medium-79 supplemented with 15% fetal calf serum, 50 µg/ml hygromycin, and 15 µg/ml G418 (7). Cultures were harvested at a density of 0.5–2 × 10⁷ cells/ml.

Identifying *T. brucei* GlnRS and GluRS—The *T. brucei* databases (tigrblast.tigr.org/er-blast/index.cgi?project=tba1 and www.sanger.ac.uk/Projects/T_brucei/) were analyzed with the BLAST software using default parameter settings to search for putative trypanosomal GlnRS and GluRS genes using the corresponding cytosolic yeast enzymes (www.yeastgenome.org/; GluRS, ORF YGL245W; GlnRS, ORF YOR168W) as query sequences. Two ORFs showing 41 and 47% identity to the yeast GlnRS and GluRS, respectively, were found. As expected for eukaryotic-type GlnRS and GluRS, the trypanosomal proteins share a high degree of sequence similarity (54%). Besides the eukaryotic-type GlnRS and GluRS, no other ORFs showing any significant similarity to either eukaryotic or bacterial-type GlnRS or GluRS or to any putative Glu-tRNA^{Gln} amidotransferase were found in the *T. brucei* genome.

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¹ The abbreviations used are: GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; RNAi, RNA interference; 2-ME, 2-mercaptoethanol; ORF, open reading frame; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.

RNA Interference—The RNAi constructs for the GlnRS and GluRS knock-down experiments are based on the previously described (7) stem loop construct carrying convenient cloning sites and a spacer corresponding to 690 nucleotides of the trypanosomal spliced leader sequence. Fragments corresponding to the 5' part of the coding region of the GlnRS ORF (28G16.175/region +33 to +619) or the GluRS ORF (43.m00224/region -4 to +476) were PCR-amplified and inserted into the corresponding sites of the modified stem loop vector. The resulting plasmids carrying one gene fragment each were then used to insert the same gene fragments in the opposite direction. The resulting RNAi plasmids were linearized with NotI and transfected into the procyclic *T. brucei* strain 29-13, which expresses T7 RNA polymerase and the tetracycline repressor (7, 8). Selection with phleomycin, cloning, and induction with tetracycline were done as described previously (9).

In Vitro Aminoacylation Using Cytosolic and Mitochondrial Extracts of *T. brucei*—RNA-free cytosolic fractions were prepared as follows. Washed *T. brucei* cells were hypotonically lysed at 1.2×10^9 cells/ml in 1 mM Tris-HCl, 1 mM EDTA, pH 8 by four passages through a 25-gauge syringe needle. A quarter volume of $5\times$ acylation buffer (250 mM Tris-HCl, pH 7.5, 125 mM KCl, 40 mM MgCl₂, 5 mM dithiothreitol) and NaCl (final concentration 150 mM) was added. The extract was centrifuged for 25 min at $150,000 \times g$ at 4 °C, and the supernatant was applied to DEAE-Sepharose equilibrated with $1\times$ acylation buffer. The flow-through fractions corresponding to RNA-free cytosol were frozen in liquid N₂ and stored at -70 °C until further use in the charging assays. Mitochondrial fractions were prepared as follows: mitoplasts of *T. brucei* were hypotonically purified as described previously (10), resuspended in 50- μ l aliquots at a concentration of ~40 mg/ml in 20 mM Tris-HCl, pH 8, 250 mM sucrose, and 2 mM EDTA, and frozen in liquid N₂. The purity of the mitoplast fraction was tested by immunoblots using antibodies against the mitochondrial heat shock protein 60 and the cytosolic protein pyruvate kinase as described previously (5). For charging assays, the mitoplasts were thawed and centrifuged for 5 min at $8,000 \times g$ at 4 °C. The pellet was resuspended in $1\times$ acylation buffer containing 1% CHAPS and spun for 5 min at $16,000 \times g$ at 4 °C. The resulting supernatant fraction (5–20 μ l) was used for the charging assays. No attempt was made to remove endogenous mitochondrial tRNAs, because very little charging was observed in the absence of added tRNAs. Aminoacylation was performed as described previously (5) using the indicated amounts of protein fractions and ~4 μ g of isolated total trypanosomal tRNA each.

Cloning of the *T. brucei* GlnRS and GluRS Genes and Expression of the Gene Product—Oligonucleotides were synthesized, and DNAs were sequenced by the Keck Foundation Biotechnology Resource Laboratory at Yale University. The gene sequences of the *T. brucei* GlnRS (28G16.175) and GluRS (43.m00224) were found at the *T. brucei* GeneDB (www.genedb.org/genedb/trypan/index.jsp). The genes were cloned by PCR from *T. brucei* genomic DNA using the Expand High Fidelity PCR system (Roche Applied Science). The cloning and subsequent expression of the full-length *T. brucei* GlnRS ORF yielded an insoluble protein; therefore, the predicted mitochondrial targeting sequence was removed (+1 to +129 in 28G16.175). An N-terminal His₆ tag was introduced into the truncated *T. brucei* GlnRS by PCR, and cloning into a pET15b expression vector (Novagen, Madison, WI) was achieved by adding a 5'-NcoI and 3'-NdeI restriction sites. Oligonucleotide primers introduced a 5'-NdeI site and a 3'-XhoI site into the full-length *T. brucei* GluRS ORF. A C-terminal His₆ tag was added by cloning the gene into the pET20b vector (Novagen, Madison, WI). All of the proteins were expressed in the *Escherichia coli* BL21-CodonPlus (DE3)-RIL strain (Stratagene, La Jolla, CA), which also expresses minor *E. coli* tRNA genes from a chloramphenicol-resistant vector. Cultures of *E. coli* transformed with either the *T. brucei* GlnRS or GluRS expression vectors were grown at 37 °C in LB medium (5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl, pH 7.0) with 0.1 mg/ml ampicillin and 0.034 mg/ml chloramphenicol. At a cell density of $A_{600} = 0.8$, the temperature was reduced to 30 °C, ampicillin was refreshed to 0.1 mg/ml, and recombinant protein expression was induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside. Upon induction, the LB medium was brought to 1% ethanol to reduce the formation of inclusion bodies. Growth was continued for 4 h post-induction, and cells were harvested by centrifugation.

Enzyme Purification—The His₆-tagged proteins were purified over a nickel-nitrilotriacetic acid column as described previously (11). GlnRS was further purified using an ÄKTA-fast protein liquid chromatography system (Amersham Biosciences) by ion-exchange chromatography on Mono S HR 5/5 (Amersham Biosciences). The pure GlnRS fraction was dialyzed into 50 mM Tris-HCl, pH 7.5, 25% glycerol, 5 mM 2-mercaptoethanol (2-ME), 0.05 mM phenylmethylsulfonyl fluoride, and 0.05

mM benzamidine-HCl, and stored at -80 °C. The GluRS was further purified on a 5-ml HiTrap heparin HP column (Amersham Biosciences) followed by gel filtration on Superdex 200 HR 10/30 (Amersham Biosciences) and then prepared for storage as described above. The enzyme fractions appeared to be >95% pure as judged by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (data not shown). Protein concentrations were determined by the Quantigold (Diversified Biotech, Boston, MA) colloidal gold protein assay (12).

Preparation of *T. brucei* tRNA—Total *T. brucei* RNA (3.95 mg) was dissolved in 1 ml of 0.1 M MOPS, pH 7.0, 1 mM EDTA, and 10 mM 2-ME and loaded onto a Qiagen-tip 100 column (Qiagen Inc., Valencia, CA) pre-equilibrated with 20 ml of 50 mM MOPS, pH 7.0, 15% isopropyl alcohol, 1% Triton-X, and 5 mM 2-ME. After washing the column with 20 column volumes of 50 mM MOPS, pH 7.0, 0.2 M NaCl, and 5 mM 2-ME, the tRNA was eluted with 2 column volumes of 50 mM MOPS, pH 7.0, 0.75 M NaCl, 15% ethanol, and 5 mM 2-ME. Further deacylation of tRNA and collection of tRNA was performed as described previously (13). This preparation yielded 223 μ g of unfractionated tRNA, which represented ~6% of the total *T. brucei* RNA used as starting material. Purity of the tRNA was >95% as determined by denaturing 12% polyacrylamide, 8 M urea gel electrophoresis.

Aminoacylation Assays—[¹⁴C]Gln (50 μ Ci/ml, 242 mCi/mmol) and [¹⁴C]Glu (50 μ Ci/ml, 254 mCi/mmol) were from Amersham Biosciences. Assays were performed essentially as described previously (14). Aminoacylation reactions were performed at 37 °C in 100 mM HEPES-Na, pH 7.2, 60 mM KCl, 30 mM MgCl₂, 10 mM ATP, 10 mM dithiothreitol, 10 μ M total *T. brucei* tRNA, and 50 μ M [¹⁴C]Gln or [¹⁴C]Glu using 100 μ M GlnRS or GluRS. The control reactions lacked tRNA or contained 60 μ M of commercially available *E. coli* total tRNA (Roche Applied Science). Sample workup and scintillation counting were carried out as before (14). The identity of the amino acids attached to tRNAs was confirmed by thin layer chromatography (data not shown) (1).

RESULTS

Analysis of the available *T. brucei* genome (www.genedb.org) by BLAST searches revealed the lack of the genes encoding the key enzyme of the transamidation pathway, Glu-tRNA^{Gln} amidotransferase, whereas single genes encoding eukaryotic-type GlnRS as well as a GluRS were detected. MITOPROT analysis predicted the presence of an N-terminal mitochondrial import signal for the *T. brucei* GlnRS and GluRS (probabilities are 0.96 [GlnRS] and 0.92 [GluRS]). This is not the case for other orthologous eukaryotic synthetases. Additional characteristics of the *T. brucei* GlnRS and GluRS are discussed below. Taken together, the genome search predicted that direct acylation should generate Gln-tRNA and that single GlnRS and GluRS enzymes should catalyze the formation of their cognate aminoacyl-tRNAs in the cytoplasm and in the mitochondria of *T. brucei*.

To test these predictions, we wanted to know whether the GlnRS and GluRS homologues are essential for the survival of *T. brucei* and where in the cell the gene products are localized. Furthermore, it was important to confirm the predicted biochemical activities of the two proteins and to investigate whether they are responsible for all of the Gln-tRNA and Glu-tRNA formation in both intracellular compartments or whether independent redundant pathways may exist.

To answer the questions outlined above, we made use of RNAi. This is the process in which the presence of a double-stranded RNA in a cell causes specific degradation of the corresponding mRNA. RNAi was shown to be a powerful method for the inhibition of gene expression in *T. brucei* (6–8, 15). It is optimally suited for RNAi analysis because as a unicellular eukaryote *T. brucei* is not prone to the recently recognized problems of RNAi analysis in mammalian cells, which are caused by the activation of interferon-mediated processes (16, 17). Therefore, unlike in mammalian cells, long double-stranded RNAs can be used to induce RNAi. We therefore established two transgenic *T. brucei* cell lines allowing tetracycline-inducible expression of double-stranded RNA corre-

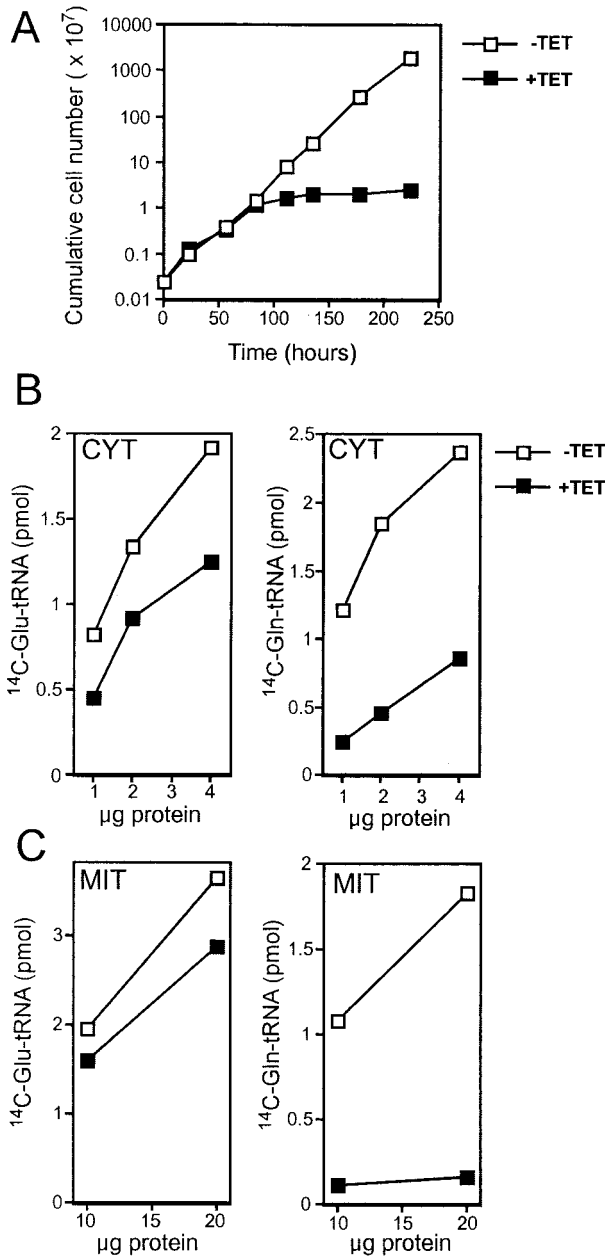


FIG. 1. Characterization of growth and GlnRS/GluRS activities in RNAi cell lines down-regulated for GlnRS. A, growth curves of the GlnRS RNAi cell line uninduced (grown without tetracycline, *-TET*) and induced for the expression of double-stranded RNA (grown in the presence of tetracycline, *+TET*). B, *in vitro* aminoacylation reactions using the indicated amounts of RNA-free cytosolic fractions (CYT) from uninduced (*-TET*) and induced (*+TET*) cells as a source of enzyme and total tRNA of *T. brucei* and [¹⁴C]Gln (right panel) or [¹⁴C]Glu (left panel) as substrates. The background corresponding to the aminoacylation activity measured in the absence of added tRNAs (approximately 5% of the total activity) was determined for each reaction and subtracted. C, same as B but detergent extracts of mitochondrial fractions (MIT) prepared from uninduced and induced cells were used as a source of enzyme.

spending to the 5' regions of the putative GlnRS and GluRS genes, respectively.

RNAi-induced Ablation of *T. brucei* GlnRS—The ablation of the GlnRS homologue in *T. brucei* results in a growth arrest ~90 h after the addition of tetracycline (Fig. 1A). Therefore, as would be expected for a translation factor, the GlnRS homologue is essential for the survival of insect stage *T. brucei*. To measure the biochemical phenotype of the RNAi cell line, we isolated an RNA-free cytosolic fraction from untreated cells and

from cells grown in the presence of tetracycline for 90 h. The cytosolic fractions were then tested for the presence of GlnRS and GluRS activities by *in vitro* aminoacylation assays using labeled glutamine or glutamate and tRNAs isolated from total cells as substrates (5). The results showed that the cytosolic fractions from both uninduced and induced cells in which the GlnRS was targeted still exhibit GluRS activity (Fig. 1B, left panel), although a slight reduction to ~70% is observed in induced cells. The GlnRS activity in induced cells, however, drops to 15–30% of the one measured in the uninduced cytosol (Fig. 1B, right panel). Thus, these results show that the putative GlnRS gene encodes the trypanosomal enzyme responsible for most if not all of the cytosolic Gln-tRNA formation.

How can the slight decline in GluRS activity in a cell line expected to be specifically down-regulated for the GlnRS activity be explained? We think that this might be due to the fact that although down-regulation of the GlnRS activity is the primary phenotype of the cell line, the resulting arrest of protein synthesis will nonspecifically affect many other physiological processes. This explanation is supported by the fact that concomitant with the drop of GluRS activity a reduction of methionyl-tRNA synthetase activity to 80% of the one found in uninduced cells was observed (data not shown). The low level of GlnRS activity that persists in the induced RNAi cell lines is not unexpected, because RNAi is known to greatly reduce the targeted mRNAs but generally does not eliminate them completely. The extent of the down-regulation depends on many factors and appears to be gene-specific (15). Trypanosomal GlnRS is predicted to contain an N-terminal mitochondrial targeting signal, which raises the question whether it also functions in mitochondria. To investigate this possibility, we isolated mitochondria from uninduced and induced (90 h) cells. The mitochondrial preparations were then used for *in vitro* charging assays. The results (Fig. 1C) are essentially identical to the ones obtained with the cytosol and demonstrate that in *T. brucei* the same gene encodes both cytosolic and mitochondrial GlnRS. Furthermore, because essentially all of the GlnRS activity was abolished in mitochondria of induced cells, these results also show that direct acylation by GlnRS is the major and probably the only pathway for the formation of mitochondrial glutaminyl-tRNA.

RNAi-induced Ablation of *T. brucei* GluRS—The results for the RNAi cell line ablated for the trypanosomal GluRS homologue are summarized in Fig. 2. The growth curve (Fig. 2A) shows that GluRS is an essential protein whose depletion results in a growth arrest reminiscent of the one seen in the GlnRS RNAi cell line. The same is true for the biochemical phenotype (Fig. 2, B and C). As expected, the GluRS activity is down-regulated while the GlnRS activity remains unaffected. These data show that a eukaryotic-type GluRS encoded by a single nuclear gene is responsible for all of the cytosolic and mitochondrial GluRS activity that was detected in *T. brucei*.

A comparison of the two knock-down cell lines shows that the specific activities for the two aminoacyl-tRNA synthetases, measured in either the cytosolic or the mitochondrial fractions, is similar in both cell lines. Furthermore, it was observed that depletion of GluRS occurs faster and that RNAi inhibition appears to be more efficient than the one observed for GlnRS.

Recombinant *T. brucei* GlnRS and GluRS Have the Predicted *In Vitro* Activities—To confirm the identities of the *T. brucei* GlnRS and GluRS, recombinant proteins were overexpressed in *E. coli* and purified to >95% homogeneity (data not shown). Total *T. brucei* tRNA was aminoacylated *in vitro* with *T. brucei* GlnRS and GluRS. These enzymes clearly charged their respective cognate amino acids onto total *T. brucei* tRNA (Fig. 3). Each enzyme reached an aminoacylation plateau of ~2.5%.

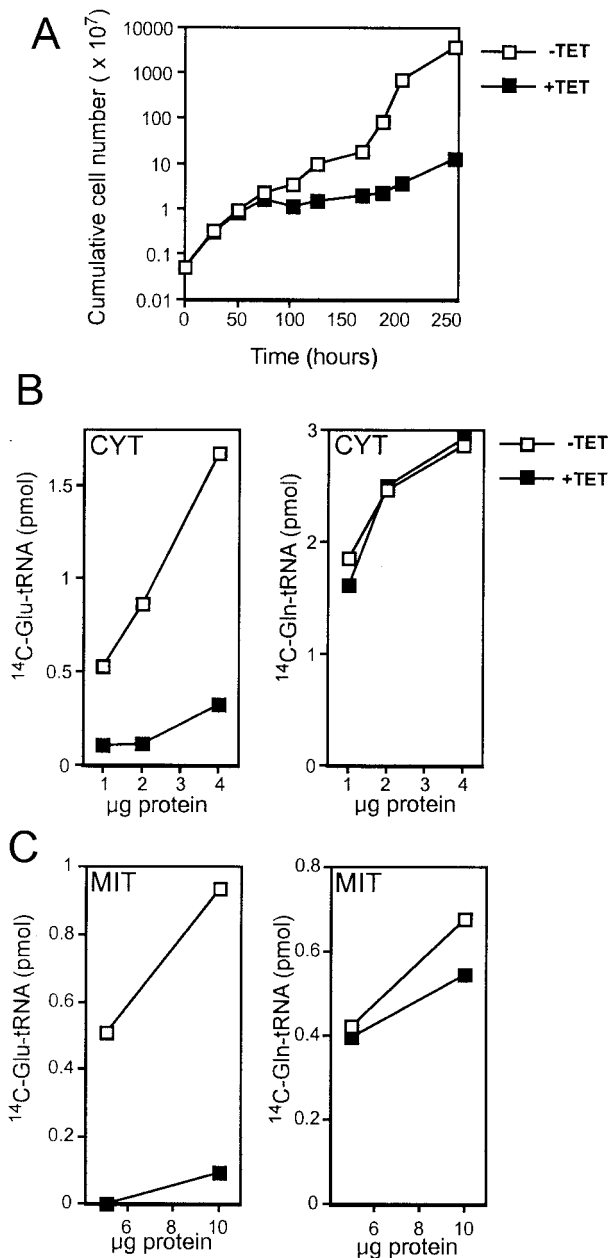


FIG. 2. Characterization of growth and GlnRS/GluRS activities in RNAi cell lines down-regulated for GluRS. *A*, growth curves of the GluRS RNAi cell lines uninduced (grown without tetracycline, -TET) and induced for the expression of double-stranded RNA (grown in the presence of tetracycline, +TET). *B*, *in vitro* aminoacylation reactions using the indicated amounts of RNA-free cytosolic fractions (CYT) from uninduced (-TET) and induced (+TET) cells as a source of enzyme and total tRNA of *T. brucei* and [¹⁴C]Gln (right panel) or [¹⁴C]Glu (left panel) as substrates. The background corresponding to the aminoacylation activity measured in the absence of added tRNAs (approximately 5% of the total activity) was determined for each reaction and subtracted. *C*, same as *B* but detergent extracts of mitochondrial fractions (MIT) prepared from uninduced and induced cells were used as a source of enzyme.

This charging level is consistent with the amount of tRNA^{Gln} and tRNA^{Glu} present in total tRNA of *T. brucei* and of other organisms (11, 18, 19). The fact that both synthetases charged unfractionated tRNA to a similar level may be a reflection of the discrimination properties of trypanosomal GluRS. Non-discriminating GluRSs, which attach glutamate to tRNA^{Gln} in addition to tRNA^{Glu}, obviously attach approximately twice as much glutamate to total tRNA (19). This property, which still needs to be rigorously established for the *T. brucei* enzyme, is

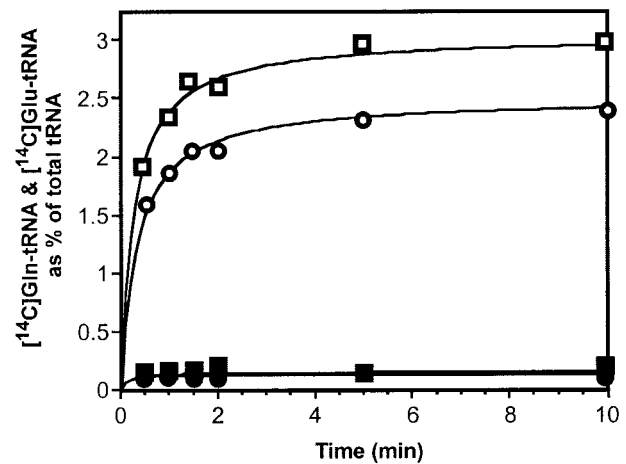


FIG. 3. Recombinant *T. brucei* GlnRS and GluRS and their respective aminoacylation activities. Aminoacyl-tRNA formation ([¹⁴C]Gln-tRNA and [¹⁴C]Glu-tRNA) is shown as the percent of total tRNA from *T. brucei* or *E. coli*. The enzyme [¹⁴C]amino acid and tRNA used are as follows: ○, *T. brucei* GlnRS, Gln, *T. brucei* tRNA; □, *T. brucei* GluRS, Glu, *T. brucei* tRNA; ●, *T. brucei* GlnRS, Gln, *E. coli* tRNA; ■, *T. brucei* GluRS, Glu, *E. coli* tRNA. The level of charging in the absence of tRNA was the same as with *E. coli* tRNA (data not shown).

essential for their role in the transamidation pathway of Gln-tRNA formation (1, 20). Thus, the *T. brucei* GluRS is probably a discriminating eukaryotic-type GluRS with 47% identity to the discriminating GluRS from yeast cytoplasm. The eukaryotic nature of the *T. brucei* GlnRS and GluRS enzymes is also seen in their inability to utilize total *E. coli* tRNA as a substrate (Fig. 3).

T. brucei GlnRS and GluRS Lack N-terminal Extensions—As depicted schematically in Fig. 4, eukaryotic GlnRS and GluRS generally have N-terminal extensions (from 123 to 264 amino acids) with regions of sequence conservation (21, 22). These extensions appear to be dispensable for aminoacylation in yeast, but the human GlnRS requires the N-terminal extension for catalytic activity (21, 23, 24). It has been suggested that the N-terminal extensions are essential for protein-protein interactions, which form the multi-aminoacyl-tRNA synthetase complexes described in yeast and higher eukaryotes (21, 23, 25). The need to form a multi-aminoacyl-tRNA synthetase complex may also explain that the GluRS of higher eukaryotes is encoded by a large open reading frame consisting of an in-frame fusion of GluRS and prolyl-tRNA synthetase (26). Not much is known yet regarding the function of these higher order aminoacyl-tRNA synthetase complexes, but in mice they are essential (27). The surprising feature of *T. brucei* GlnRS and GluRS is that they lack extensive N-terminal domains. In addition, the *T. brucei* GluRS is a smaller protein, which lacks the ProRS and linker domains found in higher eukaryotes (25). Instead, *T. brucei* GlnRS and GluRS possess short (44 and 36 amino acids, respectively) N-terminal regions predicted to be mitochondrial targeting sequences. Preliminary sequence data (ORF LmjF15.1440) show that *Leishmania major* also encodes a GlnRS with a similarly short N-terminal extension as does the metazoan parasite *Schistosoma mansoni* (28). The lack of the N-terminal domains suggests that the GlnRS and/or GluRS in these organisms do not form a higher order complex.

DISCUSSION

Using a combination of RNAi-based *in vivo* studies and *in vitro* aminoacylation assays with recombinant proteins, we have shown that direct acylation by a eukaryotic-type GlnRS is the route for mitochondrial Gln-tRNA formation in *T. brucei*. In line with this result, we find that the mitochondrial GluRS is of

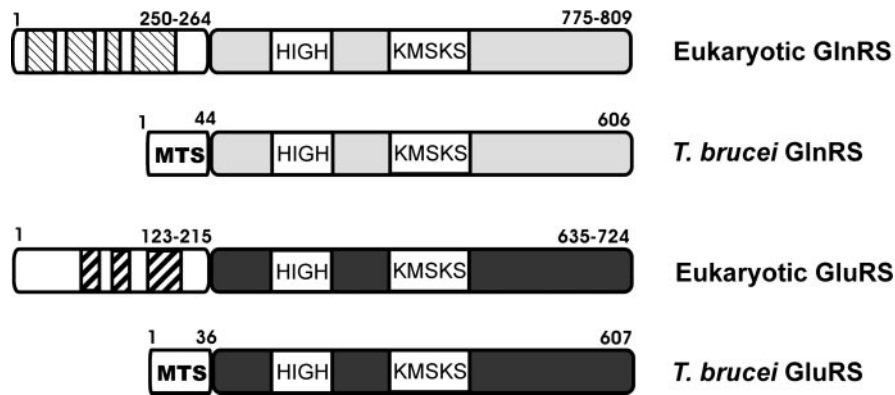


FIG. 4. **Schematic alignment of eukaryotic GlnRS and GluRS sequences.** The *T. brucei* GlnRS and GluRS are compared with a composite of eukaryotic GlnRS sequences for human (gi:1351170), mouse (gi:28546094), *Drosophila* (gi:12643524), and *Saccharomyces cerevisiae* (gi:2507436) and GluRS sequences for human (gi:135104), mouse (gi:20833867), *Drosophila* (gi:135103), and *S. cerevisiae* (gi:1711626). Only the GluRS domain of the higher eukaryotic GluProRS proteins is represented here. Lengths in number of amino acids are labeled above each segment. Sequence similarities present in the N-terminal domains are indicated by *distinct shadowing*. MTS, predicted mitochondrial targeting signal. Class I synthetase signature sequences HIGH and KMSKS are shown.

the discriminating eukaryotic type. This is expected since the presence of a bacterial-type non-discriminating GluRS would be harmful in the absence of the transamidation pathway. Interestingly, in both cases, single nuclear genes for GlnRS and GluRS encode both the cytoplasmic and the mitochondrial activities. Dual targeting of proteins to the cytosol and mitochondria is not unusual. It was also described for aminoacyl-tRNA synthetases (29, 30). However, this is the first reported case involving GlnRS and GluRS. Different mechanisms by which proteins are simultaneously targeted to the cytosol and mitochondria have been described previously (31), but currently we do not know which one is operating in *T. brucei*.

T. brucei lacks mitochondrial tRNA genes, and its mitochondrial tRNAs consist of an imported fraction of the cytosolic pool. This leads to the paradoxical situation that mitochondrial translation based on bacterial-type ribosomes must proceed exclusively with imported eukaryotic-type tRNAs (32). The use of eukaryotic-type GlnRS and GluRS to aminoacylate the eukaryotic-type tRNA^{Gln} and tRNA^{Glu} may therefore represent an adaptation to this situation. Besides *T. brucei*, there are two other organisms in which mitochondrial GlnRS activity has been detected. *Leishmania tarentolae* mitochondria also form Gln-tRNA by direct acylation (5), and the mitochondrial translation depends exclusively on imported eukaryotic-type tRNAs. However, there are some interesting differences between the two species. *T. brucei* probably has a single tRNA^{Gln}_{UUG} isoacceptor, a fraction of which is imported into mitochondria (18). In addition, as shown in this work, it has a single dually targeted GlnRS. The situation is more complex in *L. tarentolae* where two tRNA^{Gln} isoacceptors exist: (i) tRNA^{Gln}_{UUG}, which is in part imported into mitochondria, and (ii) tRNA^{Gln}_{CUG}, which appears to be cytosol-specific (33). In line with this finding, it was shown that *L. tarentolae* contains distinct cytosolic and mitochondrial GlnRS activities exhibiting distinct substrate specificities toward the two isoacceptors (5). However, the genes encoding the GlnRS activities have not been identified, and the lack of RNAi methodologies in *Leishmania* (34) has hampered a more detailed investigation. Mitochondrial GlnRS activity was also detected in *Tetrahymena* mitochondria (35). The *Tetrahymena* mitochondrial genome encodes only a subset of tRNAs, and the mitochondrial tRNA^{Gln} species is imported from the cytosol (36). The type of GlnRS(s) that exists in *Leishmania* and *Tetrahymena* mitochondria has not been determined; however, based on our results in *T. brucei*, they can be expected to be of the eukaryotic type.

The observed correlation between the presence of mito-

chondrial GlnRS activity and the mitochondrial import of its tRNA^{Gln} substrate suggests that mitochondrial GlnRS might be limited to organisms that import eukaryotic-type tRNA^{Gln} from the cytosol. Eukaryotes with mitochondria-encoded tRNA^{Gln} may use the transamidation pathway or possess a bacterial-type GlnRS. The bacterial ancestor of mitochondria probably had a complete set of tRNA genes. A lack of mitochondrial tRNA genes and import of cytosolic tRNAs is therefore a derived trait (4). This finding suggests that the mitochondrial GlnRS activity was only acquired later in evolution.

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