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Mitochondrial tRNA Import in *Toxoplasma gondii**

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Apicomplexan parasites have the smallest known mitochondrial genome. It consists of a repeated element of ~6–7 kb in length and encodes three mitochondrial proteins, a number of rRNA fragments, but no tRNAs. It has therefore been postulated that in apicomplexans all tRNAs required for mitochondrial translation are imported from the cytosol. To provide direct evidence for this process we have established a cell fractionation procedure allowing the isolation of defined organellar RNA fractions from the apicomplexan *Toxoplasma gondii*. Analysis of *T. gondii* total and organellar RNA by Northern hybridization showed that except for the cytosol-specific initiator tRNA^{Met} all nucleus-encoded tRNAs tested were present in the cytosol and in the mitochondrion but not in the plastid. Thus, these results provide the first experimental evidence for mitochondrial tRNA import in apicomplexans. The only other taxon that imports the whole set of mitochondrial tRNAs are the trypanosomatids. Interestingly, the initiator tRNA^{Met} is the only cytosol-specific tRNA in trypanosomatids, indicating that the import specificity is identical in both groups. In agreement with this, the *T. gondii* initiator tRNA^{Met} remained in the cytosol when expressed in *Trypanosoma brucei*. However, in contrast to trypanosomatids, no thio- modifications were detected in the tRNA^{Gln} of *T. gondii* indicating that, unlike what is suggested in *Leishmania*, they are not involved in regulating import.

Toxoplasma gondii is an obligate intracellular parasite of humans and animals. Whereas in immunocompetent individuals a *T. gondii* infection is generally asymptomatic, immunosuppressed patients are at risk of severe disease (1). *T. gondii* and *Plasmodium falciparum*, the causative agent of human malaria, belong to the apicomplexans and are evolutionarily closely related. Thus, in addition to being an important pathogen of its own, *T. gondii* serves as a model system for *Plasmodium* to study some experimentally less accessible aspects of apicomplexan biology (2). Interestingly, apicomplexans contain a non-photosynthetic plastid whose genome shares many features with chloroplast DNA (3). As expected for eukaryotes, apicomplexans also contain mitochondria. However, although much effort has been invested to investigate the plastid, there are only very few studies on apicomplexan mitochondria. This is surprising, because their biology is not only expected to be of medical relevance but, due to some unique features, is also of great interest for basic science. It has been proposed that the contribution of mitochondrial ATP synthesis to the total cellular pool is minimal (4). However, the presence of a mitochondrial membrane potential has been demonstrated in both intracellular *T. gondii* tachyzoites (5) and intraerythrocytic *Plasmodium yoelii* (4). Furthermore, it has been suggested that the anti-apicomplexan drug atovaquone acts by interfering with the electron transport chain of the parasites through inhibition of cytochrome *b* in the bc1 complex (4). This is supported by the detection of mutated cytochrome *b* genes in the mitochondrial DNA of atovaquone-resistant *T. gondii* cell lines (6). Thus, the mitochondrial electron transport must be essential for some apicomplexan life cycle stages that are found in the vertebrate host.

The most striking difference between mitochondrion of the apicomplexan and that of another of the species is its DNA. It consists of a tandemly repeated element of ~6–7 kb and encodes subunits I and III of cytochrome *c* oxidase, cytochrome *b*, and a number of short fragments representing the small and the large subunit rRNAs (7–9). This makes it the shortest mitochondrial genome with the most limited coding capacity known. Most interestingly, the apicomplexan mitochondrial genome does not encode any tRNAs. The lack of apparently essential mitochondrial tRNA genes has been reported in other organisms, such as plants, some fungi, and other protozoa. It is known that, in these organisms, the lacking mitochondrial tRNA genes are compensated for by import of the corresponding cytosolic tRNAs (10). The number of tRNAs that are imported depends on the species. *Saccharomyces cerevisiae* mitochondria import a single tRNA only. Plants import a significant fraction of their mitochondrial tRNAs but still have mitochondria-encoded ones. The most extreme situation is found in trypanosomatids (such as *Trypanosoma brucei* and *Leishmania*) (11, 12), a group of parasitic protozoa that evolutionarily are not related with the apicomplexans but whose mitochondrial genomes, like those of apicomplexans, completely lack mitochondrial tRNA genes. Whereas nothing is known about mitochondrial tRNA import in apicomplexans, the process has been studied in quite some detail in trypanosomatids. It was shown that an imported nucleus-encoded mitochondrial tRNA always represents only a small fraction (typically 1–7%) of a normal cytosolic tRNA (13). Although all mitochondrial tRNAs derive from the cytosol, a single cytosol-specific tRNA has been identified in *T. brucei*. This tRNA corresponds to the eukaryotic initiator tRNA^{Met} (tRNA$^{Met\text{-}i}$),[1] and its cytosol-specific localiza-

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[1] The abbreviations used are: tRNA$^{Met\text{-}i}$, initiator tRNA^{Met}; tRNA$^{Met\text{-}e}$, elongator tRNA^{Met}; LSU rRNA, large subunit ribosomal RNA; MTF, tRNA^{Met}-formyl transferase.

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tion is mediated by two nucleotide pairs in the T-stem of the tRNA (14). Furthermore, results obtained in *Leishmania* suggest that cytosol-specific 2-thiouridines, which are found in the anticodon wobble position of tRNA^{Glu}(UUC) and tRNA^{Gln}(UUG), may regulate the extent of import because the two tRNAs carrying these modifications were prevented from being imported *in vitro* (15).

The aim of the present work was to directly demonstrate tRNA import in the apicomplexan *T. gondii* and to determine its specificity. Furthermore, we wanted to compare the features of mitochondrial tRNA import in *T. gondii* to the one of trypanosomatids.

EXPERIMENTAL PROCEDURES

Cells—Tachyzoites of *T. gondii* (RH strain) were maintained in Vero cell monolayers at 37 °C and 5% CO_2 in Roswell Park Memorial Institute medium containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2–10% immunoglobulin-free horse serum. Tachyozoites were harvested from their feeder cell cultures as previously described (16). The preparation containing tachyzoites and host cell debris was washed twice in cold phosphate-buffered saline and passed through a pre-equilibrated Sephadex G-25M column. The eluted purified parasites were centrifuged (3000 × *g* for 3 min at 4 °C), and the resulting pellets were used either directly, to isolate total RNA and for *in vivo* aminoacylations, or they were subjected to subcellular fractionation.

Procyclic *T. brucei* cells, stock 427, were grown at 27 °C in SDM-79 medium supplemented with 5% fetal bovine serum. Cells were harvested at late log phase, corresponding to 2.5×10^7 to 3.5×10^7 cells/ml, and washed once in cold 20 mM sodium phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose. The resulting cellular pellets were used either to isolate total RNA or to prepare mitochondria.

Subcellular Fractionation of T. gondii—Washed *T. gondii* cells (2×10^8 cells corresponding to ~0.5 mg of protein) were resuspended in 0.5 ml of SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA). Five percent of the sample (25 μl) was removed to isolate the total RNA. After the addition of 0.475 ml of SoTE containing either 0.1% (0.05% final) or 0.2% (0.1% final) of digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The suspensions were centrifuged (8000 × *g* for 5 min at 4 °C), and the supernatants were discarded. The resulting pellets were resuspended in 500 μl of SoTE containing 1 μg of RNase A and incubated on ice for 15 min to digest the contaminating cytosolic RNAs. The organellar fractions used to isolate RNA were recovered in the pellets after a final centrifugation step.

Subcellular Fractionation of T. brucei—Digitonin extraction (final concentration, 0.035%) was essentially performed as described previously (14). The obtained pellet was essentially free of cytosolic RNA and, because *T. brucei* does not have a plastid, corresponded to a crude mitochondrial fraction.

RNA Isolation and Northern Analysis—RNA from total cells or digitonin-treated fractions of *T. gondii* or *T. brucei* was purified by using the acidic guanidinium isothiocyanate method as previously described (17). The samples containing the isolated RNAs were separated on 8 M urea-10% polyacrylamide gels. A formaldehyde-agarose gel was used for the Northern analysis of the cytochrome *b* mRNA. Blotting and hybridizations using 5′-end-labeled oligonucleotides were done as described (13). Affinity gel-electrophoresis for thio-modified residues (Fig. 5) was performed on 8 M urea-10% polyacrylamide gels containing 25 μM ((*N*-acryloylamino)phenyl)mercuric chloride (18).

Hybridization Probes—Oligonucleotides recognizing the cytosolic 5.8 S rRNA (GAGCCAAGACATCCATTG) (19), the plastid tRNA^{Met} (AACCTGCTCTACCCCGCT) (20), and the mitochondrial large subunit (LSU) rRNA fragment (GACAAGGATTTTCCTACCTT) of *T. gondii* (21) were designed using published sequence information. The probe to detect mitochondrial-encoded cytochrome *b* mRNA (6) was prepared by PCR amplification using CGAGAACACTCAGTCTATC and CAGATACGTAGAAACCTCC as primers.

Oligonucleotides hybridizing to nucleus-encoded *T. gondii* tRNAs were designed using sequence information from the public *T. gondii* databases (preliminary genomic and/or cDNA sequence data were accessed via ToxoDB.org and/or www.tigr.org/tdb/t_gondii/). tRNA genes were identified by BLAST using tRNA genes from other organisms as templates. The following oligonucleotides were used: tRNA^{Ala} (TGGACGACATGGGTATCG), tRNA^{Ile} (TGGTCCCAACCGGGATCG), tRNA^{Ser} (CGACGGCGGCAGGATTCG), tRNA^{Trp} (TGAGCCCAGAGCGACTCG), tRNA^{Met-e} (CTGCGAGGATCGAACTCG), tRNA^{Met-i} (CCCACTGAGCTACGGTGC), and tRNA^{Gln} (AGGTCCCACCGGGACTCG). To detect *T. brucei* tRNAs (Fig. 4) the following probes were used: GTGGTGGTCCTACCAGGAT for tRNA^{Gln} and TGAGGACTGCAGGGATTG for tRNA^{Trp}. The oligonucleotides hybridizing to the *T. brucei* tRNA^{Met-i} and tRNA^{Met-e} are described in Ref. 14.

In Vivo Aminoacylation Assay—Washed *T. gondii* (5×10^7 cells) were resuspended in 300 μl of 1× SBG (20 mM glucose, 0.15 M NaCl, 20 mM NaP_i, pH 7.9) containing 100 μg/ml cycloheximide and 20–40 μCi of [^{35}S]methionine/[^{35}S]cysteine mixture (>1000 Ci/mmol). The labeled [^{35}S]cysteine (~25% of total) was competed out by the addition of 0.5 mM unlabeled cysteine. The reaction was incubated for 15 min at 37 °C. Intact cells were then centrifuged (3000 × *g*, for 3 min at 4 °C), and the RNA was isolated from the resulting pellet as described above.

In Vitro Aminoacylation Assay—The assays were done in 100 μl of acylation buffer (50 mM Tris-HCl, pH 7.5), 25 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 10% glycerol) containing 20–40 μCi of a [^{35}S]methionine/[^{35}S]cysteine mixture, 0.5 mM unlabeled cysteine, 2 mM ATP, RNA isolated from 10^7 *T. gondii* tachyzoites or from the digitonin fractions (2×10^8 cell equivalents each), and 30–60 μg of RNA-free cytosolic protein extract of either *T. gondii* or *T. brucei*, as sources of methionyl-tRNA synthetase activity (22). To be able to test externally added substrate tRNAs, it was essential to use protein extracts that were free of endogenous RNAs. Removal of endogenous RNA from the cytosolic fractions was achieved through binding to a DEAE-Sepharose column in the presence of 150 mM NaCl (23). Incubation was for 15 min at 37 °C, and RNAs were re-isolated as described above.

The ^{35}S-labeled tRNAs^{Met} from both the *in vivo* and the *in vitro* assays were analyzed on 8 M urea/10% polyacrylamide gels and visualized by fluorography. To prevent deacylation of the labeled tRNAs^{Met} during electrophoresis, the samples were subjected to a deamination procedure (11).

Transfection of T. brucei—For expression of wild-type and variant *T. gondii* tRNA^{Met-i} in *T. brucei* cells, derivatives of the pHD-437 plasmid (24), which allow stable integration into the trypanosomal rDNA loci, were used. The KpnI/BamHI fragment of the plasmid was replaced by inserts containing either *T. gondii* wild-type or variant genes of tRNAs^{Met}. Variations in the tRNA^{Met-i} were introduced by PCR-mediated mutagenesis and verified by sequencing. Both tRNAs^{Met-i} were expressed containing 179 nucleotides of their own 5′-flanking region and 61 nucleotides of their 3′-flanking sequence. The constructs were linearized with NotI and electroporated into *T. brucei*, and transformants were selected with phleomycine and cloned as previously described (25).

RESULTS

Characterization of T. gondii Organellar RNA Fractions—To investigate mitochondrial tRNA import in *T. gondii* it was essential to prepare defined organellar RNA fractions, which can be analyzed by Northern blots using oligonucleotides recognizing specific nucleus-encoded *T. gondii* tRNAs. BLAST searches on the available *T. gondii* genomic sequences allowed us to identify candidate tRNA genes by homology to the corresponding tRNAs in other organisms. Preparing defined organellar RNA fractions of *T. gondii* was a bigger challenge for the following reasons. It is difficult and expensive to obtain *T. gondii* cells in large quantities, and, contrary to *T. brucei*, apicomplexans in addition to mitochondria also contain a plastid that has its own genome and translation system. It was therefore necessary not only to fractionate the mitochondria from the cytosol but also to separate the two organelles from each other. Recently, we have performed an extensive analysis of mitochondrial tRNA import in *T. brucei* requiring the isolation of mitochondrial RNAs from many different cell lines (13, 14). This was achieved by using the detergent digitonin, which at the correct concentration selectively dissolves the cellular membranes but leaves (at least the inner) mitochondrial membrane(s) intact. Subsequent RNase digestion allows the removal of contaminating cytosolic RNAs. Finally, a centrifugation step results in a pellet that essentially only contains mitochondrial RNAs. The big advantage of this procedure is that it requires a relatively small number of cells only. We therefore tested whether the procedure would be suitable for apicomplexans. Purified and washed *T. gondii* tachyzoites were

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extracted with 0.05 and 0.1% of digitonin and subjected to RNase digestion. The resulting RNA fractions together with total *T. gondii* RNA were then analyzed by specific Northern hybridizations. To identify the different organellar fractions oligonucleotide probes recognizing specific marker RNAs were used. These RNAs were the 5.8 S rRNA for the cytosol and the plastid-encoded $tRNA^{Met}$ for the plastid, both of which can easily be detected on Northern blots. To choose a mitochondrial marker was more difficult, because the mitochondrial genome of *T. gondii*, unlike that of many other apicomplexans, has not been sequenced yet. However, fragments apparently derived from the mitochondrial DNA have been detected in the nuclear genome of *T. gondii* (21). These sequences appear to code for typical mitochondrially encoded proteins and show a high homology to the mitochondrial genomes of other apicomplexans but are not expressed. Southern blot hybridization furthermore showed that in addition to the nucleus these sequences also occur in an extrachromosomal, tandemly repeated element of ~6 kb, suggesting that they are also present within the *bona fide* mitochondrial genome of *T. gondii* (8). We therefore chose a segment of the nuclear mitochondrial-like sequences that is identical to a region coding for a fragment of the mitochondrial LSU rRNA in *Plasmodium* to design an oligonucleotide expected to hybridize to mitochondrial RNA only. It has been shown that specific mutations in the cytochrome *b* gene lead to atovaquone-resistant *T. gondii* cells, indicating that mitochondrial cytochrome *b* is expressed (6). Thus, in addition to the putative LSU rRNA we were using the mRNA of cytochrome *b* as a mitochondrial marker. The results of a digitonin-based organellar fractionation of *T. gondii* cells are shown in Fig. 1. Total RNA and RNA isolated from the RNase-treated pellet fractions obtained from 0.05 and 0.1% digitonin extractions were tested for the presence of cytosolic and organellar RNAs. The *top panel* shows that as expected the cytosolic marker, 5.8 S rRNA, is detected in the total RNA fraction only. The *next two panels* show the intracellular distribution of the mitochondrial LSU rRNA fragment and cytochrome *b* mRNA, respectively. Both RNAs are recovered in the 0.05% digitonin fraction but are accessible to added nucleases in the presence of 0.1% of detergent. Thus, mitochondria with an intact membrane barrier are specifically recovered in the 0.05% digitonin fraction. The *last panel* shows that the plastid marker, the $tRNA^{Met}$, remains protected from added RNase in both pellet fractions, indicating that the plastid membranes (at least the innermost), unlike the mitochondrial ones, remain intact even after the 0.1% digitonin extraction. Hence, 0.05% digitonin extraction and subsequent RNase digestion yield a fraction containing mitochondrial and plastid RNAs, which is essentially free of intact cytosolic RNAs. Extraction with 0.1% digitonin, in contrast, results in a fraction containing plastid RNAs only.

Nucleus-encoded T. gondii tRNAs Are Imported into Mitochondria—Fig. 1*B* shows the intracellular distribution of four nucleus-encoded *T. gondii* tRNAs. All were detected in the total RNA fraction as well as in the 0.05% digitonin pellet but were absent from the 0.1% pellet. These results show that, although the largest fraction of each of the tRNAs is found in the cytosol (note that the total RNA fraction essentially corresponds to the cytosol), a small but significant part is recovered in the mitochondria. Quantitatively between 1.5 and 2% of the total cellular content of each tRNA is localized within the mitochondrion (Fig. 1*C*). These numbers are in the same range as those for *T. brucei* where 1–7% of a given tRNA is localized within mitochondria (13).

*Localization of T. gondii tRNAs^Met^*—A global analysis of mitochondrial tRNA import in *T. brucei* has shown that, with a single exception, all trypanosomal tRNAs are in part imported

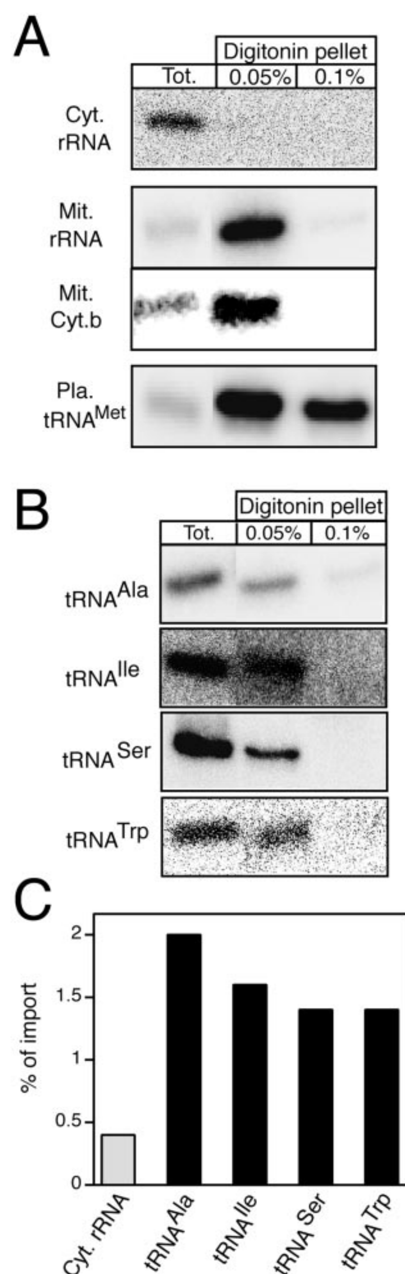

FIG. 1. **Isolation of organellar RNA fractions of *T. gondii* and intracellular localization of nucleus-encoded tRNAs.** *A*, Northern blot analysis of total RNA (*Tot.*), and RNA obtained by 0.05% and 0.1% digitonin extractions. *Top panel*: blot probed for the cytosolic 5.8 S rRNA. *Middle panels*: duplicate blots probed for a mitochondrial LSU rRNA fragment (*Mit. rRNA*) and mitochondrial cytochrome *b* mRNA (*Mit. Cyt b*), respectively. *Bottom panel*: duplicate blot probed for the plastid-encoded $tRNA^{Met}$ (*Pla.* $tRNA^{Met}$). Note that for the total RNA, 1×10^7 cell equivalents and for the digitonin fractions, 2×10^8 cell equivalents (20-fold more) were loaded. *B*, duplicate blots (same as *A*) were probed for nucleus-encoded $tRNA^{Ala}$, $tRNA^{Ile}$, $tRNA^{Ser}$, and $tRNA^{Trp}$. *C*, quantification of hybridization signals from the Northern blots shown in *B*. The extent of import per cell was calculated and expressed as % of the total RNA, which is found in mitochondria. The signals on Northern blots were quantified in the linear response range. The cytosolic cross-contamination is shown in *gray*, whereas the percentage of mitochondrial import is shown in *black* for each individual tRNA.

into mitochondria (13). The only tRNA behaving differently is the $tRNA^{Met-i}$, which is exclusively found in the cytosol. It was therefore of great interest to analyze the intracellular localization of the nucleus-encoded *T. gondii* $tRNAs^{Met}$. As expected BLAST searches of the *T. gondii* genomic sequences identified

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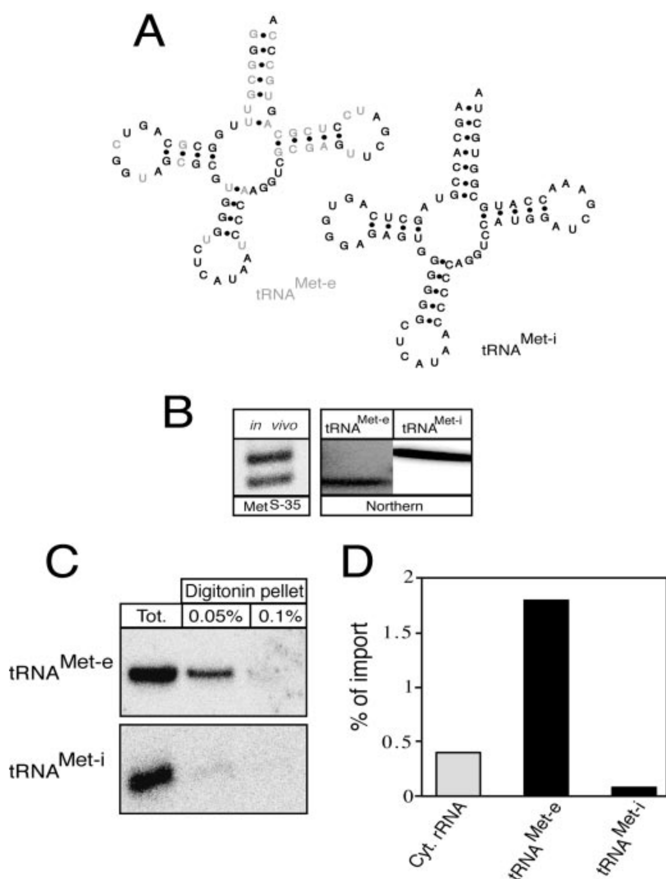

FIG. 2. **Expression and intracellular localization of tRNAs^{Met}.** *A*, predicted secondary structures of *T. gondii* tRNA^{Met-e} and tRNA^{Met-i}. Nucleotides that are different between the two molecules are shown in *gray* in the tRNA^{Met-e}. *B*, two tRNAs^{Met} are expressed *in vivo*. *Left panel*: *in vivo* aminoacylation assays using [^{35}S]methionine. *Right panel*: Northern blot containing total cellular RNAs probed for the predicted tRNA^{Met-e} and tRNA^{Met-i}, respectively. The *left* and the *right parts* of this panel represent two stripes from the same preparative lane. *C*, intracellular localization of *T. gondii* tRNAs^{Met}. RNA isolated from total cells (*Tot.*), and from the 0.05% or 0.1% digitonin fractions were probed for tRNA^{Met-e} and tRNA^{Met-i}, respectively. Loadings are the same as in Fig. 1*A* and 1*B*. *D*, quantification of import using the hybridization signals from Fig. 2*C*. Calculations were done as for Fig. 1*C*.

two distinct but homologous tRNAs^{Met}, one corresponding to the elongator tRNA^{Met} (tRNA^{Met-e}) and the other one corresponding to the eukaryotic tRNA^{Met-i} (Fig. 2*A*). In agreement with this we found two [^{35}S]methionine-labeled bands in an *in vivo* aminoacylation experiment (Fig. 2*B*, *left lane*), suggesting that only two cytosolic tRNA^{Met} exist in *T. gondii*. Furthermore, comparison with a Northern blot analysis run on a duplicate gel and using oligonucleotides designed to specifically detect either of the two tRNAs^{Met} allowed us to attribute the upper band to the tRNA^{Met-i} and the lower one to the tRNA^{Met-e}. Using the same oligonucleotides we also analyzed the RNA fraction from both total cells and from digitonin-extracted samples. The results in Fig. 2*C* show that the tRNA^{Met-e} is imported into mitochondria and therefore behaves the same as all the other tRNAs tested before (Fig. 1*B*). However, reminiscent of the situation in *T. brucei*, the *T. gondii* tRNA^{Met-i} remained in the cytosol. The quantification in Fig. 2*D* shows that less of the tRNA^{Met-i} is found in the mitochondrial fraction than of the cytosolic marker, the full-length 5.8 S rRNA. This can be explained by the fact that tRNAs are expected to be soluble molecules, whereas the 5.8 S rRNA is a component of ribosomes and some of which may remain associated with mitochondria.

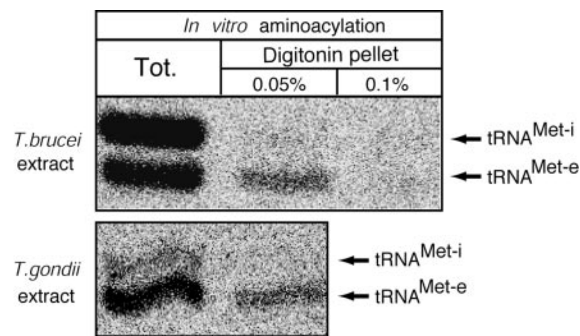

FIG. 3. ***In vitro* aminoacylation using [^{35}S]methionine.** Aminoacylation assays were performed by using either cytosolic extract of *T. brucei* (*top panel*) or cytosolic extract of *T. gondii* (*bottom panel*) as source of methionyl-tRNA synthetase activities. *T. gondii* RNAs isolated from total cells (*Tot.*), and from 0.05% or 0.1% digitonin fractions were used as substrates. Positions of tRNA^{Met-i} and tRNA^{Met-e} are indicated by *arrows*.

In a further analysis we subjected aliquots of total and digitonin-extracted organellar RNA fractions to *in vitro* aminoacylation assays using radioactive [^{35}S]methionine and either RNA-free cytosolic extract of *T. brucei* or *T. gondii* as a source of enzyme. As expected and in agreement with the *in vivo* results shown in Fig. 2*B*, for both extracts two labeled bands, corresponding to tRNA^{Met-i} and tRNA^{Met-e}, were detected in the total RNA fraction (Fig. 3, *left lanes*). In the 0.05% digitonin pellet containing the mitochondrial RNAs, only the lower band corresponding to the tRNA^{Met-e} was observed (Fig. 3, *middle lanes*). Thus, these results confirm the cytosol-specific localization of the tRNA^{Met-i}. Furthermore, they suggest that the only tRNA^{Met} present in mitochondria of *T. gondii*, corresponds to the tRNA^{Met-e} identified in the genomic data base. The methionyl-tRNA synthetase activity in the *T. gondii* cytosolic extract was lower than the one in the *T. brucei* extract. This is explained by the fact that it was difficult to prepare a sufficiently concentrated RNA-free cytosolic fraction from *T. gondii* due to the limited amount of material that was available. However, this cannot account for the fact that the *T. gondii* methionyl-tRNA synthetase preferentially charges the tRNA^{Met-e} when tested *in vitro* (Fig. 3, *bottom panel left lane*), whereas the *T. brucei* extract preferentially charges the tRNA^{Met-i} (Fig. 3, *top panel left lane*).

There are at least two possible explanations for this observation. It could be that, although the methionyl-tRNA synthetase activities in *T. gondii* and *T. brucei* can in principle charge both *T. gondii* tRNAs^{Met}, their preferred substrate tRNAs^{Met} are different. Alternatively, it is possible that the *T. brucei* extract aminoacylates (with methionine) a *T. gondii* tRNA species other than tRNA^{Met}.

Expression of T. gondii tRNAs^{Met} in T. brucei—Expression of tRNA^{Met} variants in transgenic *T. brucei* has successfully been used to identify the determinants responsible for their intracellular localization (14). In principle the same approach should be feasible for *T. gondii*. However, despite repeated attempts, we were not able to express tRNA^{Met} variants in transgenic *T. gondii*. We therefore took the converse approach and expressed *T. gondii* tRNAs^{Met} in *T. brucei*. However, this was also problematic, because, of the many variants of *T. gondii* tRNA^{Met} that were assessed, only the wild-type tRNA^{Met-i} and a variant thereof carrying the T-arm of the tRNA^{Met-e} could be expressed. This was surprising, because we have previously successfully expressed many variants of trypanosomal (14) as well as heterologous tRNAs (26), and this suggests that for unknown reasons expression of most *T. gondii* tRNA^{Met} variants is harmful for *T. brucei*. The results of the two *T. gondii* tRNAs^{Met} that could be expressed are shown in

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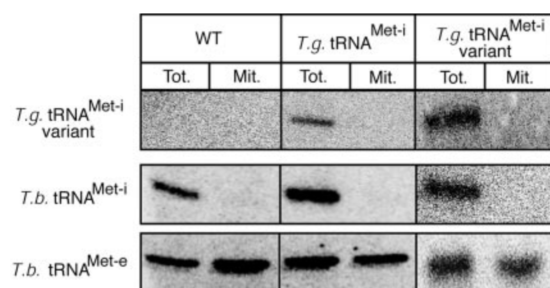

FIG. 4. **Intracellular localization of wild-type and variant *T. gondii* tRNAs^{Met-i} expressed in *T. brucei*.** Northern blot analysis of *T. brucei* wild-type cells (*WT*) and of cell lines expressing *T. gondii* tRNA^{Met-i} (*middle panel*) or the *T. gondii* tRNA^{Met-i} containing the T-arm of the *T. gondii* tRNA^{Met-e} (tRNA^{Met-i} variant) (*right panel*). RNA isolated from total cells (*Tot.*) and from mitochondrial fractions (*Mit.*) were analyzed. *Top panel*: Northern blot probed for *T. gondii* tRNA^{Met-i}. *Middle* and *bottom panels*: duplicate blots probed for the cytosol-specific tRNA^{Met-i} and the imported wild-type tRNA^{Met-e} of *T. brucei*.

Fig. 4. Total and mitochondrial RNA from wild-type and the two transgenic *T. brucei* cell lines were analyzed for the presence of the *T. gondii* tRNA^{Met-i} (Fig. 4, *middle panel*) and the tRNA^{Met-i} variant carrying the T-arm of the *T. gondii* tRNA^{Met-e} (Fig. 4, *right panel*), respectively. The *top panel* in Fig. 4 shows that in *T. brucei* both *T. gondii* tRNAs^{Met} remain in the cytosol. The *lower two panels* of Fig. 4 show the intracellular distribution of the trypanosomal wild-type tRNA^{Met-i}, which serves as the cytosolic marker, and that of the partially imported wild-type tRNA^{Met-e}, respectively. The fact that the *T. gondii* tRNA^{Met-i} remains in the cytosol is expected, because it contains the cytosolic T-stem localization determinants, which were defined for the *T. brucei* tRNAs^{Met} (14). In contrast, the cytosolic localization of the tRNA^{Met-i} variant carrying the T-arm of the *T. gondii* tRNA^{Met-e} is unexpected, because it contains the predicted trypanosomal mitochondrial T-stem localization determinants.

It is therefore possible that the localization determinants in *T. gondii* and *T. brucei* tRNAs^{Met} are different. However, we cannot exclude that the *T. gondii* tRNA^{Met-i} variant when expressed in *T. brucei* is less efficiently charged. The unexpected localization of the *T. gondii* tRNA^{Met-i} variant, therefore, does not automatically mean that incompatible localization determinants are present but could in principle be a secondary effect due to poor charging. Interestingly, however, as shown in previous experiments (26) most heterologous elongator tRNAs behave differently and generally are imported into trypanosomal mitochondria indicating that, at least for elongator tRNAs, import is the default pathway.

Absence of Thio-modified Nucleotides in T. gondii tRNA^{Gln} and tRNA^{Trp}—In trypanosomatids all tRNAs, with the exception of the tRNA^{Met-i}, are imported into the mitochondria to variable extents, and as shown in this work the same appears to be true for *T. gondii*. This raises the question of how the extent of import is regulated. Recently, it was suggested that in *Leishmania*, this is achieved by cytosol-specific 2-thiouridines, which are found in the anticodon wobble position of leishmanial tRNA^{Glu} and tRNA^{Gln} and which act as anti-import determinants (15). To see whether this might also be the case in apicomplexans we analyzed the *T. gondii* tRNA^{Gln} for the presence of thio-modified nucleotides by using affinity gel electrophoresis for thiolated residues (18). The result of such an analysis for the *T. brucei* tRNA^{Gln} is shown in the *left panel* of Fig. 5. A retardation of the band, which is specific for the cytosolic fraction, was observed indicating that the cytosolic but not the mitochondrial tRNA^{Gln} contains a thio-modified nucleotide. The converse result was obtained for the *T. brucei* tRNA^{Trp}, part of which was selectively thio-modified in the mitochondrial fraction only (Fig. 5, *bottom left panel*). These results are identical to the ones obtained for *Leishmania*, where cytosol- and mitochondria-specific thiolation of the tRNA^{Gln} (15) and tRNA^{Trp} (27), respectively, have first been described and suggest that the pattern of thio- modifications is conserved within trypanosomatids. Interestingly, a very different result is obtained for *T. gondii* tRNA^{Gln} and tRNA^{Trp}. Both tRNAs were lacking any thio-modified nucleotides, irrespective of whether they were isolated from the cytosol or from mitochondria. Thus, in *T. gondii*, unlike that suggested in trypanosomatids, thio-modified nucleotides cannot be involved in regulating the extent of tRNA^{Gln} import. Furthermore, the fact that thio-modified nucleotides were also absent from the imported tRNA^{Trp} may indicate a general absence of thio-modified tRNAs in *T. gondii*.

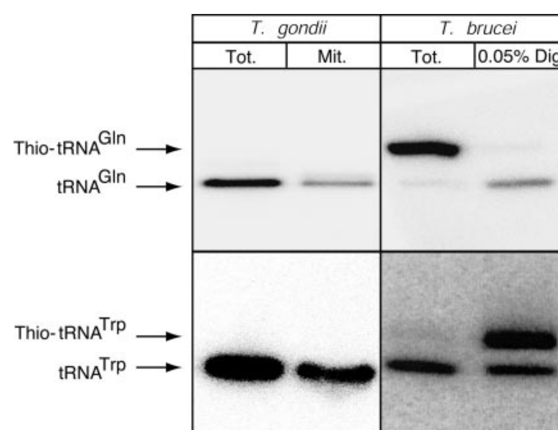

FIG. 5. **Absence of thio-modified tRNAs in *T. gondii*.** Northern blots containing total (*Tot.*) and mitochondrial RNA (*Mit.* and 0.05% *Dig.*) from *T. brucei* (*left panel*) and *T. gondii* (*right panel*), respectively, were probed for the presence of the *T. brucei* and *T. gondii* tRNA^{Gln} (*top panel*) and tRNA^{Trp} (*bottom panel*), respectively. The samples were resolved on an 8 M urea/10% polyacrylamide gel containing 25 μM of ((*N*-acryloylamino)phenyl)mercuric chloride, which retards the migration of thio-modified molecules. *Arrows* indicate thio-modified and unmodified molecules.

DISCUSSION

The mitochondrial genome of apicomplexans lacks tRNA genes (7). It has therefore been postulated that these organisms import all their tRNAs from the cytosol. Using defined organellar RNA fractions, obtained by a newly established fractionation procedure (Fig. 1*A*), we have tested this prediction for the apicomplexan parasite *T. gondii*. Six out of six nucleus-encoded elongator-type tRNAs (tRNA^{Ala}, tRNA^{Ile}, tRNA^{Ser}, tRNA^{Trp}, tRNA^{Gln}, and tRNA^{Met-e}) tested were in part recovered in the mitochondrial fraction (Figs. 1*B*, 2*C*, and 5) and therefore imported from the cytosol. This suggests that in *T. gondii* a small fraction of any given cytosolic elongator-type tRNA is imported into mitochondria. Interestingly, however, the nucleus-encoded eukaryotic-type tRNA^{Met-i} only occurs in the cytosol. tRNA^{Met-i} and tRNA^{Met-e} differ only by 31 nucleotides (Fig. 2*A*) indicating that these differences or a subset thereof must be responsible for their differential localization. This situation is reminiscent of the one in the trypanosomatid *T. brucei*, which also imports all mitochondrial tRNAs (13). Furthermore, the only trypanosomal cytosol-specific tRNA, just as in *T. gondii*, corresponds to the cytosolic tRNA^{Met-i}. It differs from the imported tRNA^{Met-e} by 26 nucleotides, and a recent study (14) has shown that two nucleotide pairs in the T-stem region are both necessary and sufficient to specify the localization of the trypanosomal tRNAs^{Met}.

Imported tRNAs are always of the eukaryotic type (10). Thus, we have the extraordinary situation in *T. gondii* and

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T. brucei mitochondria of a bacterial-type translation system that has to function with imported eukaryotic type tRNAs only. Although most eukaryotic type tRNAs can be expected to function in the context of a bacterial type translation system, this is not the case for tRNAs^{Met}. All organisms have two classes of tRNAs^{Met}, an tRNA$^{Met\text{-}i}$, which is used for initiation of protein synthesis, and a tRNA$^{Met\text{-}e}$, which functions in the insertion of methionine into internal peptidic linkages. Whereas elongator tRNAs$^{Met\text{-}e}$ of all organisms look similar, there are two distinct groups of tRNAs$^{Met\text{-}i}$. The tRNA$^{Met\text{-}i}$ of the eukaryotic cytosol carries an A1:T72 base pair, which is not found in any other tRNA. Bacterial and organellar tRNAs$^{Met\text{-}i}$, on the other hand, are characterized by a mismatch at the top of the acceptor stem and carry a formylated methionine (28, 29). Thus, it makes sense that the only cytosol-specific tRNA detected in *T. gondii* as well as *T. brucei* corresponds to the eukaryotic-type tRNA$^{Met\text{-}i}$, because this tRNA could not possibly function in the context of the bacterial type translation systems of mitochondria. However, the fact that the imported tRNA$^{Met\text{-}e}$ appears to be the only tRNA^{Met} present in mitochondria raises the question of how mitochondrial translation initiation in apicomplexans and trypanosomatids, known to require a formylated bacterial type tRNA$^{Met\text{-}i}$, can function with an imported tRNA$^{Met\text{-}e}$.

Mitochondrial translation initiation has been studied in detail in *T. brucei*, and it was shown that a fraction of the imported tRNA$^{Met\text{-}e}$ becomes formylated after import into mitochondria and subsequently can act in translation initiation (22, 30). Formylation of a tRNA$^{Met\text{-}e}$ is very unusual and requires a distinct tRNA^{Met} formyl-transferase (MTF). The enzyme has been identified in *T. brucei* and was shown to have a diametrically opposed substrate specificity compared with all other known bacterial and organellar MTFs, in that it selectively recognizes tRNA$^{Met\text{-}e}$. Furthermore, the trypanosomal protein is approximately twice the size of all other MTFs (22).

Do apicomplexan mitochondria also use formylated tRNA$^{Met\text{-}e}$ to initiate mitochondrial translation? Due to the limited amount of mitochondrial RNA available it was not possible to analyze the formylation state of the imported tRNA$^{Met\text{-}e}$ in *T. gondii*. However, BLAST searches of the two complete and annotated genomes of *P. falciparum* and *Plasmodium yoelli* show that these two apicomplexans possess a single MTF, each of which has approximately twice the size of MTFs from other organisms. Preliminary analysis of the as yet unannotated genome suggests that also *T. gondii* has an unusually large MTF. Thus, it is possible that, just as in *T. brucei*, the apicomplexan MTF formylates imported tRNA$^{Met\text{-}e}$ and that the large molecular weight of the proteins is linked to their unusual substrate specificity. However, the problem with this explanation is that apicomplexans appear to have a single MTF gene only. This is unexpected, because both mitochondria and the plastid are expected to require formylated tRNA^{Met} for organellar translation initiation. It is therefore possible that the identified apicomplexan MTF genes encode the plastid enzymes or that the proteins are targeted to both organelles. Examples for dual targeting of proteins to the plastid and to mitochondria have been described before (31). Finally, taking into account that only three proteins are synthesized in the mitochondria of apicomplexans (8), it is also conceivable that the need for a formylated tRNA^{Met} for translation initiation has been bypassed altogether. Thus, the question of which tRNA is used for mitochondrial translation initiation at present remains open.

In summary, we present for the first time experimental evidence for mitochondrial tRNA import in apicomplexans and show that the specificity of the process is identical to the one in trypanosomatids. Apicomplexans and trypanosomatids are not closely related, which raises the question, whether the uncovered similarities of the two systems are due to a remote common ancestor or arose independently through convergent evolution. Answering this question will require a detailed knowledge of the tRNA import mechanisms in both organisms.

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