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A Nuclear Encoded tRNA of *Trypanosoma brucei* Is Imported into Mitochondria

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The mitochondrial genome of trypanosomes, unlike that of most other eukaryotes, does not appear to encode any tRNAs. Therefore, mitochondrial tRNAs must be either imported into the organelle or created through a novel mitochondrial process, such as RNA editing. Trypanosomal $tRNA^{Tyr}$, whose gene contains an 11-nucleotide intron, is present in both the cytosol and the mitochondrion and is encoded by a single-copy nuclear gene. By site-directed mutagenesis, point mutations were introduced into this tRNA gene, and the mutated gene was reintroduced into the trypanosomal nuclear genome by DNA transfection. Expression of the mutant tRNA led to the accumulation of unspliced $tRNA^{Tyr}$ (A. Schneider, K. P. McNally, and N. Agabian, J. Biol. Chem. 268:21868–21874, 1993). Cell fractionation revealed that a significant portion of the unspliced mutant $tRNA^{Tyr}$ was recovered in the mitochondrial fraction and was resistant to micrococcal nuclease treatment in the intact organelle. Expression of the nuclear integrated, mutated tRNA gene and recovery of its gene product in the mitochondrial fraction directly demonstrated import. In vitro experiments showed that the unspliced mutant $tRNA^{Tyr}$, in contrast to the spliced wild-type form, was no longer a substrate for the cognate aminoacyl synthetase. The presence of uncharged tRNA in the mitochondria demonstrated that aminoacylation was not coupled to import.

The transport of nuclear encoded RNAs into mitochondria has been implicated in a variety of higher plants and protozoa (5, 16). These RNAs are tRNAs and can represent either a small subset (higher plants) (13) or the whole set (trypanosomes and *Leishmania* spp.) (8, 19) of mitochondrial tRNAs. tRNA import has also been postulated for *Saccharomyces cerevisiae*, even though the yeast organellar genome encodes all tRNAs necessary for protein synthesis. For the latter, hybridization data suggest that a single nuclear encoded tRNA, $tRNA^{Lys}$, is imported into the mitochondria. The function of the imported $tRNA^{Lys}$, however, is unclear, as this tRNA cannot be aminoacylated by the mitochondrial charging enzyme (14, 23). The evidence for mitochondrial RNA import is based almost exclusively on indirect data: the hybridization of mitochondrial RNAs to nuclear DNA but not to mitochondrial DNA and the inability to find a complete set of tRNA genes in the mitochondrial genome. An alternative explanation for these data is possible: the existence of RNA editing in plant and trypanosomal mitochondria (20, 25) has made it impossible to rule out the possibility that mitochondrial tRNAs are created by extensive modification of transcripts from cryptic mitochondrial genes. This is not just a formal possibility, since the editing of mitochondrial tRNAs was recently shown for *Acanthamoeba castellanii* (11). There is only one species, potato, in which RNA import was confirmed by more direct genetic methods: transgenic potato plants carrying a heterologous $tRNA^{Leu}$ gene from bean nuclear DNA were shown to contain RNA transcribed from the introduced gene in both the cytosolic fraction and the mitochondrial fraction (21).

The tRNA may or may not be complexed with proteins during import. In the first case, it may use its own import system, whereas in the second case, it may be imported through the protein import channel. Recently, in vitro import into yeast mitochondria of an oligonucleotide chemically cross-linked to an artificial precursor protein was reported (24). This result shows that in principle, the protein import machinery is able to transport nucleic acids. In *Phaseolus vulgaris* and *Tetrahymena thermophila*, identical aminoacyl synthetases appear to be responsible for charging both the nuclear encoded imported tRNAs and their cytosolic counterparts, suggesting that these synthetases may act as cotransporters (7, 22).

Trypanosoma brucei provides an excellent system with which to study mitochondrial RNA import, since evidence suggests that not just a few but all of the tRNAs are imported (8) and since genetic techniques can easily be applied (1, 6). We developed an in vivo system for assaying mitochondrial RNA transport and used it to validate the hypothesis that tRNAs encoded in the nucleus are imported into mitochondria and not created inside mitochondria by RNA editing.

MATERIALS AND METHODS

Strains. The wild-type strain used was the procyclic form of *T. brucei* IsTat 1.1. The transformed strain was previously derived from IsTat 1.1 by stable transformation with plasmid pHyg-Sup as the transfecting DNA. The transformant contains four point mutations in the anticodon stem-loop of one of the two $tRNA^{Tyr}$ gene alleles (18).

Isolation of mitochondria. Mitoplast and cytosolic fractions were prepared from 6 liters (1×10^7 to 2×10^7 cells per ml) each of the wild-type cell line or the transformed cell line by published procedures (10). Cells were lysed under hypotonic conditions by passage through a hypodermic needle (26 gauge) and centrifuged at 6,000 × *g*. An aliquot of the supernatant was used to prepare cytosolic RNA. The pellet was suspended in isotonic buffer to 4×10^9 cell equivalents per ml and digested with DNase I (final concentration, 10 μg/ml) and micrococcal nuclease (final concentration, 50 U/ml). After an additional centrifugation at 6,000 × *g*, the pellet was sus-

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pended in isotonic buffer containing 60% Percoll and fractionated on a 20 to 35% Percoll floating gradient.

RNA isolation. RNA was purified by the acid guanidinium isothiocyanate method as described elsewhere (3).

Northern (RNA) analysis. RNAs were electrophoretically separated on an 8 M urea–10% polyacrylamide gel, and Northern analysis was performed as described previously (18). All radiolabeled probes were hybridized at a high stringency, corresponding, in all cases, to a 50°C hybridization temperature. The sequences of the oligonucleotides were as follows: 5′CCCGCATGATTTAGAGTC3′ for the probe complementary to nucleotides 29 to 46 of unspliced mutant $tRNA^{Tyr}$ and 5′CCTGTGATCTACAGTCAC3′ for the probe hybridizing to nucleotides 28 to 45 of mature wild-type $tRNA^{Tyr}$. 5′AG GAGATAGGACTTGCCCT3′ anneals to mitochondrial 9S rRNA, and 5′GCCATCACTGATCGCCGTAGTAAC3′ specifically binds to cytosolic 5S rRNA.

Micrococcal nuclease treatment. Freshly isolated mitoplasts (1.5×10^{10} cell equivalents) were suspended in 0.3 ml of 20 mM Tris-HCl (pH 8.0)–2 mM EDTA–250 mM sucrose–50% (wt/vol) glycerol–1 mg of bovine serum albumin per ml (fatty acid free) and divided into three equal samples. All samples were adjusted to 3 mM $CaCl_2$. After the addition of Triton X-100 to the third sample (1% [wt/wt] final concentration) and 15,000 U of micrococcal nuclease to the second and third samples, each was incubated for 20 min at 23°C. The digestion was terminated by the addition of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) to a final concentration of 10 mM. RNA from all samples was purified by the acid guanidinium isothiocyanate method as described elsewhere (3), except that 10 μg of glycogen was added as a carrier before ethanol precipitation.

Aminoacylation and deamination of tRNAs. Ten micrograms of total RNA isolated from the wild-type or the transformed cell line was aminoacylated with 100 U of a crude mixture of yeast synthetases (Sigma) and 20 μCi of [^{3}H]tyrosine in 50 mM Tris-HCl (pH 7.65)–10 mM potassium chloride–5 mM magnesium acetate–2 mM ATP–2 μM each amino acid except for tyrosine for 30 min at 37°C. The amino acid moiety of the aminoacyl-tRNA was deaminated according to published procedures (8, 19). Deamination is necessary to stabilize the aminoacyl-tRNA bond and prevent deacylation at pH 8.0 during gel electrophoresis. The samples were electrophoresed on an 8 M urea–10% acrylamide gel and visualized by fluorography. As a control, to visualize charged and uncharged $tRNA^{Tyr}$s, duplicate samples were aminoacylated with nonradioactive tyrosine and, after deamination, analyzed on a Northern blot with a radiolabeled oligonucleotide probe directed against the 3′ end of the tRNA (5′GTGGTCCTTCCG GCCGGAATCGAA3′).

RESULTS

Genetic approach. As an experimental approach, to directly demonstrate mitochondrial RNA import, *T. brucei* was transfected with a marked copy of its own $tRNA^{Tyr}$ gene, whose transcript was previously shown to be present in both the cytosol and the mitochondrion. $tRNA^{Tyr}$ was chosen because, unlike most other tRNAs, it is encoded by a single-copy gene (15). To discriminate the tRNA transcribed from the transfected gene from the endogenous one, it was necessary to tag its gene by mutations. Since $tRNA^{Tyr}$ contains an intron (12, 18), mutations which led to the accumulation of unspliced precursor tRNA were introduced (18). The modified gene product could therefore be identified both by its different size and, because more than one point mutation was introduced, by specific oligonucleotide hybridization.

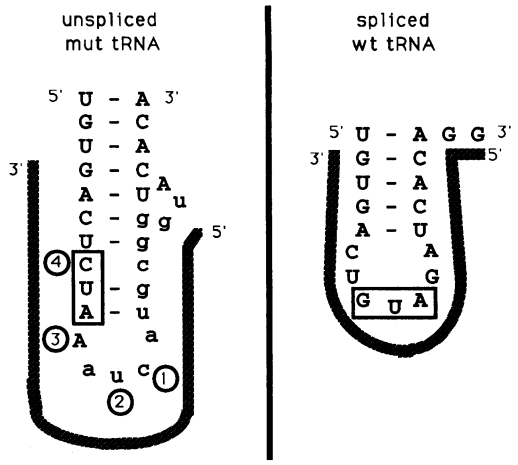

FIG. 1. Putative secondary structures of the anticodon loops of the two major $tRNA^{Tyr}$ populations detected in the transformed strain; the anticodons are boxed. $tRNA^{Tyr}$ transcribed from the transformed gene, shown on the left (mut tRNA), contains an intron (lowercase letters) and four mutations, numbered 1 to 4. Numbers 1 (U-to-C substitution) and 2 (G-to-U substitution) were placed within the intron sequence, number 3 (G-to-A substitution) was placed at the splice junction nucleotide on the 5′ exon, and number 4 (G-to-C substitution) is an amber-suppressing transversion at the 5′ position of the $tRNA^{Tyr}$ anticodon. This mutation abolishes splicing, resulting in the accumulation of unspliced tRNA (18). On the right, the anticodon loop of spliced wild-type $tRNA^{Tyr}$ is shown (wt tRNA). Oligonucleotide probes indicated by the shaded lines (see Materials and Methods) allowed for the specific detection of unspliced mutant or mature wild-type $tRNA^{Tyr}$ when hybridized at a high stringency.

Southern blot analyses showed that the transformed mutant gene was integrated into the nuclear genome and replaced one $tRNA^{Tyr}$ allele, leaving the other copy unaltered. No integration into mitochondrial DNA was observed (data not shown). Figure 1 shows the two forms of $tRNA^{Tyr}$ which were expressed in the transformed cells, one corresponding to 3′-end, processed, unspliced molecules transcribed from the mutant $tRNA^{Tyr}$ allele and the other being spliced $tRNA^{Tyr}$ originating from the wild-type allele. No mature form transcribed from the transformed gene could be detected (18).

Unspliced mutant $tRNA^{Tyr}$ cofractionates with mitoplasts. To test whether the mutant gene product is imported into mitochondria, cytosolic and mitochondrial fractions were prepared from wild-type and transformed cells. The fractionation procedure involves initial hypotonic lysis of the trypanosomes, which disrupts the outer membrane of the mitochondria and converts them into mitoplasts (2, 10). This fact was confirmed by measuring the activity of inner membrane-localized succinate-cytochrome *c* reductase. The activity of that enzyme did not change when assayed in the presence or absence of detergent, indicating the absence of an intact outer membrane (data not shown). RNA extracted from cytosolic and mitoplast fractions was analyzed by Northern hybridizations with specific oligonucleotide probes. Unspliced mutant tRNA was detected uniquely in the transformed strain, in which it was found in both the cytosol and the mitoplasts (Fig. 2A); mature wild-type tRNA was detected in all fractions in both cell types. The ratios of $tRNA^{Tyr}$ in the cytosol versus the mitoplasts were comparable for mature wild-type and unspliced mutant $tRNA^{Tyr}$s (Fig. 2B). Cross-contamination of the fractions was negligible,

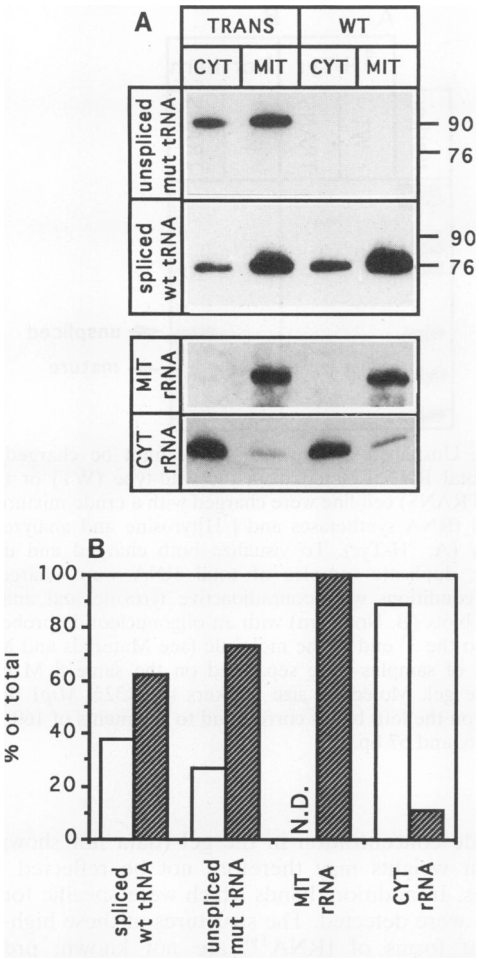

FIG. 2. Unspliced mutant $tRNA^{Tyr}$ originating from the transfected gene was found in both the cytosol and the mitoplasts. (A) Quadruplicate Northern blots containing 0.8 μg of cytosolic RNA (CYT) and 4 μg of RNA extracted from mitoplasts (MIT) from either wild-type (WT) or transformed (TRANS) cells were hybridized with oligonucleotide probes (see Materials and Methods) that discriminate between unspliced mutant $tRNA^{Tyr}$ (mut tRNA) and spliced wild-type $tRNA^{Tyr}$ (wt tRNA); 9S rRNA (MIT rRNA) and 5S rRNA (CYT rRNA) were used as mitochondrial and cytosolic markers, respectively. Molecular size markers (in base pairs) (pBR322, *Msp*I digest) are indicated on the right. (B) The signals detected in the transformed strain were quantitated by densitometric scanning. The total additive signal obtained from the cytosol (0.8 μg) (□) and the mitoplasts (4 μg) (▨) was set at 100% for each probe. N.D., not detected.

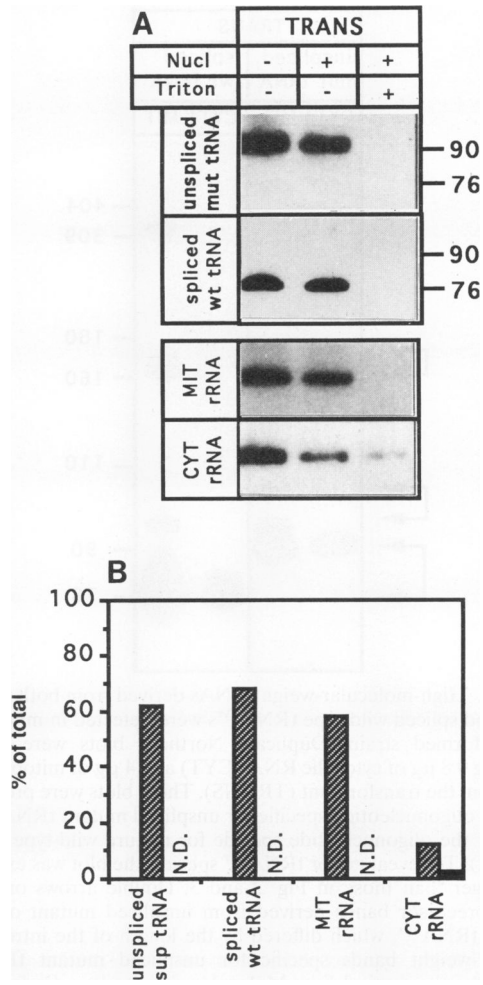

FIG. 3. Unspliced mutant $tRNA^{Tyr}$ found in the mitoplast fraction was localized inside mitoplasts. (A) Mitoplasts originating from transformed cells (TRANS) either were left untreated or were incubated with micrococcal nuclease (Nucl) in the presence or absence of 1% Triton X-100 (Triton), and their respective RNAs were separated on an 8 M urea–10% polyacrylamide gel. Duplicate Northern blots of the RNAs were probed for unspliced mutant $tRNA^{Tyr}$ (mut tRNA) and mature wild-type $tRNA^{Tyr}$ (wt tRNA). Blots were subsequently reprobed for 9S rRNA (MIT rRNA) as a mitochondrial marker or 5S rRNA (CYT rRNA) as a cytosolic marker with specific oligonucleotide probes (see Materials and Methods). Molecular size markers (in base pairs) (pBR322, *Msp*I digest) are indicated on the right. (B) The autoradiographs were quantitated by densitometric scanning. Results were normalized with untreated samples set at 100%. Hatched bars represent micrococcal nuclease-treated samples (no Triton X-100). No signals were detected in samples incubated with micrococcal nuclease in the presence of 1% Triton X-100 (N.D., not detected), except for 5S rRNA (CYT rRNA), for which 2% of the input RNA was nuclease and Triton X-100 resistant (black bar).

as assessed by hybridization with probes specific for mitochondrial 9S rRNA and cytosolic 5S rRNA. These experiments demonstrate that a significant amount of unspliced mutant $tRNA^{Tyr}$, like its mature wild-type counterpart, fractionates with mitochondria.

Unspliced mutant $tRNA^{Tyr}$ is localized in the mitochondrial matrix. If unspliced mutant $tRNA^{Tyr}$ is indeed transported across mitochondrial membranes, it should resist enzymatic hydrolysis in intact mitoplasts. Approximately 60% of both mutant and wild-type $tRNA^{Tyr}$s from the mitoplast fraction was found to be resistant to micrococcal nuclease treatment, unless the mitochondrial inner membrane was disrupted with 1% Triton X-100 (Fig. 3). Since a similar percentage of the mitochondrial 9S rRNA was also resistant, we presume that the 40% signal reduction was due to mitoplast leakage during the incubation. Figure 3A, bottom panel, shows that the small amount of cytosolic 5S rRNA which contaminated mitochondria was reduced by 90%. Since mitochondria fragment and reseal during the hypotonic isolation procedure (2, 10), the remaining 5S rRNA found in nuclease-treated mitoplasts may have reflected molecules which became trapped during organellar fractionation. Microscopic examination revealed no evidence for contaminating nuclei in the organellar fraction. In addition, the pores present in the nuclear membrane allow free

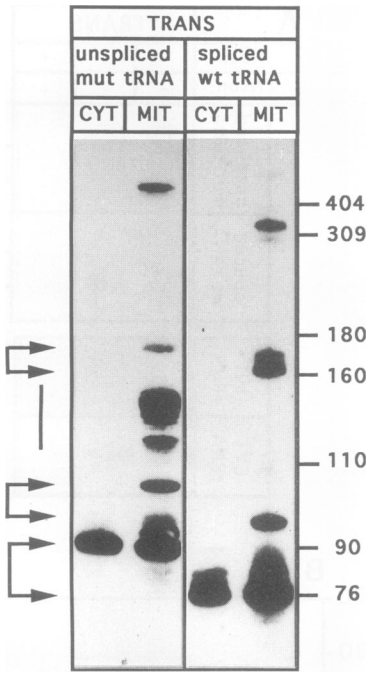

FIG. 4. High-molecular-weight RNAs derived from both unspliced mutant and spliced wild-type $tRNA^{Tyr}$s were detected in mitoplasts of the transformed strain. Duplicate Northern blots were prepared containing 0.8 μg of cytosolic RNA (CYT) and 4 μg of mitoplast RNA (MIT) from the transformant (TRANS). These blots were probed with either the oligonucleotide specific for unspliced mutant $tRNA^{Tyr}$ (mut tRNA) or the oligonucleotide specific for mature wild-type $tRNA^{Tyr}$ (wt tRNA). To reveal minor $tRNA^{Tyr}$ species, the blot was exposed 10 times longer than those in Fig. 2 and 3. Double arrows on the left indicate precursor bands derived from unspliced mutant or mature wild-type $tRNA^{Tyr}$, which differed by the length of the intron. High-molecular-weight bands specific for unspliced mutant tRNA are indicated by the vertical line. Molecular size markers (in base pairs) (pBR322, *Msp*I digest) are indicated on the right.

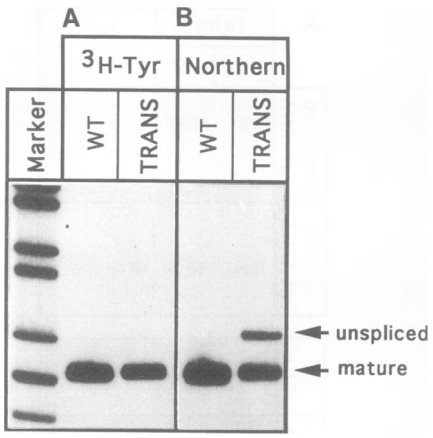

FIG. 5. Unspliced mutant $tRNA^{Tyr}$ cannot be charged with tyrosine. Total RNA isolated from the wild-type (WT) or the transformed (TRANS) cell line were charged with a crude mixture of yeast aminoacyl tRNA synthetases and [^{3}H]tyrosine and analyzed by fluorography (A, ^{3}H-Tyr). To visualize both charged and uncharged $tRNA^{Tyr}$s, duplicate samples of total RNA were charged under identical conditions with nonradioactive tyrosine and analyzed on Northern blots (B, Northern) with an oligonucleotide probe complementary to the 3′ end of the molecule (see Materials and Methods). Both sets of samples were separated on the same 8 M urea–10% acrylamide gel. Molecular size markers (pBR322, *Msp*I digest) are indicated on the left; bands correspond to fragments of 160, 147, 123, 110, 90, 76, and 67 bp.

diffusion of low-molecular-weight proteins, like micrococcal nuclease, so nuclear RNAs would be digested. This suggestion was confirmed by Northern analysis, in which no U6 small nuclear RNA, a nuclear marker, could be detected in the mitoplast fraction (data not shown). It is therefore concluded that nuclease-resistant unspliced mutant $tRNA^{Tyr}$ is localized inside the inner membrane in the matrix of the mitoplasts and consequently represents imported molecules.

Mitoplasts contain high-molecular-weight forms of $tRNA^{Tyr}$. Extended exposures of Northern blots containing cytosolic and mitochondrial RNAs and probed with oligonucleotides specific for either spliced wild-type or unspliced mutant $tRNA^{Tyr}$ revealed high-molecular-weight forms of $tRNA^{Tyr}$ which were restricted to the mitoplast fraction (Fig. 4). The existence of mitoplast-specific high-molecular-weight forms of $tRNA^{Tyr}$ in transformed cells, which contained the mutations introduced in vitro, supports the conclusion that the molecules found in mitoplasts are imported into the organelle and do not represent cytosolic contaminants. A set of bands detected with a probe specific for unspliced mutant $tRNA^{Tyr}$ exhibited a similar pattern but with a slightly decreased mobility when compared with the wild-type forms of $tRNA^{Tyr}$ (Fig. 4, double arrows); this shift was most likely due to the presence of the intron. The set of bands showing the lowest electrophoretic mobility ran aberrantly, depending on the acrylamide concentration in the gel (data not shown); their molecular weights may therefore not be reflected by their mobilities. In addition, bands which were specific for mutant $tRNA^{Tyr}$ were detected. The structures of these high-molecular-weight forms of $tRNA^{Tyr}$ are not known; preliminary primer extension analyses suggest that they contain 5′ extensions of the tRNA (data not shown).

Unspliced mutant $tRNA^{Tyr}$ cannot be charged with tyrosine. The cotransport model postulates that tRNAs are imported as complexes with proteins. Possible candidates for such cotransporters are the cognate synthetases. It has therefore been suggested that aminoacylation of tRNA may be coupled to its import (22). The import of unspliced mutant $tRNA^{Tyr}$ in the transformed cell line has clearly been established; it was therefore of interest to find out whether the mutant tRNA could still be charged. To do so, total RNA was isolated from either the wild-type cell line or the transformed cell line and charged in vitro with tritiated tyrosine and commercially available yeast aminoacyl synthetases. Mature wild-type $tRNA^{Tyr}$ present in both the wild-type and the transformed cells could easily be charged. Unspliced mutant $tRNA^{Tyr}$ however, could not be aminoacylated under identical conditions (Fig. 5A). The presence of unspliced molecules in the transformed strain was confirmed by Northern analyses of duplicate samples charged under identical conditions with nonradioactive tyrosine (Fig. 5B). Unspliced mutant $tRNA^{Tyr}$ is therefore not a substrate for aminoacyl synthetase, most likely as a result of the presence of the intron, which changes the anticodon loop structure. Alternatively, the introduction of the four base-pair changes may interfere with recognition of the tRNA by the charging enzyme. The experiment was performed with heterologous yeast synthetases, but since $tRNA^{Tyr}$s from *S. cerevisiae* and trypanosomes are highly homologous, it is unlikely that this fact accounts for the results. The presence in mitochondria of a tRNA which cannot be

charged supports the hypothesis that in trypanosomes, aminoacylation is not required for import.

DISCUSSION

Evidence for tRNA import into mitochondria is quite widespread; it has been postulated for a number of plants, some protozoa, and even *S. cerevisiae* (5, 14, 16). However, nothing is known about the signals and the mechanism of this process because almost all evidence for mitochondrial RNA import is based on indirect data. In only one system, *Solanum tuberosum*, has import directly been proven by genetic transformation with a heterologous tRNA gene (21). The production of transgenic plants is tedious and time-consuming; therefore, a system which is easier to manipulate would be of great advantage for the study of mitochondrial RNA import. The protozoan *T. brucei* provides such a system; it can easily be genetically manipulated, and stable, transient, and plasmid-based transformations are possible (1, 6, 17). Trypanosomes have the additional advantage that the entire set of mitochondrial tRNAs is postulated to be imported.

We have established an in vivo system for studying mitochondrial RNA import in *T. brucei*: introduction of a mutagenized $tRNA^{Tyr}$ gene into the trypanosomal nuclear genome by homologous recombination and recovery of the predicted mutant gene product inside mitoplasts directly prove the import of tRNA into the mitochondrion and exclude the alternative explanation that this tRNA is created by extensive modification of a transcript from a cryptic mitochondrial gene. Unspliced mutant and spliced wild-type $tRNA^{Tyr}$s behave similarly in terms of localization, nuclease sensitivity in isolated mitoplasts, and the presence of mitoplast-specific high-molecular-weight forms, indicating that both mutant and wild-type forms are imported. The data also show that both mitochondrial and cytosolic $tRNA^{Tyr}$s in *T. brucei* are encoded by the same nuclear gene. Whereas the present data demonstrate that $tRNA^{Tyr}$ is imported into mitochondria, the possibility cannot be excluded that other mitochondrial tRNAs are created by RNA editing.

The fact that an intron-containing tRNA is imported is unexpected, since splicing is a nuclear process (4). It appears that mutant $tRNA^{Tyr}$ does not interact with the splicing machinery and therefore is able to escape from the nucleus to the cytoplasm. Once in the cytoplasm, it is imported into the mitochondrion with an efficiency comparable to that of spliced wild-type $tRNA^{Tyr}$. This fact indicates that the import system tolerates significant deviations from the classic tRNA structure, at least within the anticodon loop.

High-molecular-weight forms of both spliced wild-type and unspliced mutant $tRNA^{Tyr}$s were detected exclusively in the mitochondrial fraction. This finding is consistent with the results reported by Hancock et al. (9) describing a population of long tRNA molecules in *T. brucei* mitochondria that were converted to mature forms by treatment with *E. coli* RNase P. The structure and sequence of the high-molecular-weight forms of $tRNA^{Tyr}$ are not known. Primer extension analyses have indicated that these forms are in part due to 5′ extensions. The speculation has been that these high-molecular-weight molecules represent precursor forms of $tRNA^{Tyr}$ and that the 5′ extensions may target them to mitochondria. The in vivo import system offers an excellent tool for testing this hypothesis.

In vitro experiments show that unspliced mutant $tRNA^{Tyr}$ cannot be charged with tyrosine by use of yeast synthetases. The substrate used in our in vivo import system therefore represents an uncharged tRNA, assuming that the unspliced $tRNA^{Tyr}$ is not being charged in vivo in the homologous system. In contrast, for *S. tuberosum*, direct evidence for the import of a heterologous tRNA has been presented. That tRNA, however, could still be charged in vitro (21). It therefore appears that in trypanosomes, the charging of tRNA is not coupled to mitochondrial import.

The in vivo import system is easy to manipulate and should allow us to identify the structural and sequence requirements for tRNA trafficking between the nucleus and the mitochondrion. Genetic transformation of trypanosomal mitochondria is currently impossible; in vivo import of RNAs may therefore provide an alternative tool for manipulating mitochondrial gene expression, for example, by use of antisense RNAs. Such an approach would require the insertion of defined foreign sequences into the imported tRNAs. The experiments presented in this study show that in principle this approach is feasible, since the import of a mutated intron sequence of 11 nucleotides, which is not found in the mitochondria of wild-type cells, has been achieved.

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