Nucleic Acids Research, 1994, Vol. 22, No. 18 3699–3705

Nuclear-encoded mitochondrial tRNAs of *Trypanosoma brucei* have a modified cytidine in the anticodon loop

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Received June 9, 1994; Revised and Accepted August 10, 1994

ABSTRACT

The mitochondrial genome of *Trypanosoma brucei* does not appear to encode any tRNA genes. Isolated organellar tRNAs hybridize to nuclear DNA, suggesting that they are synthesized in the nucleus and subsequently imported into the mitochondrion. Most imported tRNAs have cytosolic counterparts, showing identical mobility on two-dimensional polyacrylamide gels. We have compared three nuclear-encoded mitochondrial tRNAs ($tRNA^{Lys}$, $tRNA^{Leu}$, $tRNA^{Tyr}$) with their cytosolic isoforms by direct enzymatic sequence analysis. Our findings indicate that the primary sequences of the mitochondrial and the corresponding cytosolic tRNAs are identical. However, we have identified a mitochondrion-specific nucleotide modification of each tRNA which is localized to a conserved cytidine residue at the penultimate position 5′ of the anticodon. The modification present in mature mitochondrial $tRNA^{Tyr}$ was not found in a mutant $tRNA^{Tyr}$ defective in splicing in either cytosolic or mitochondrial fractions. The mutant $tRNA^{Tyr}$ has been expressed in transformed cells and its import into mitochondria has been demonstrated, suggesting that the modified cytidine residue is not required for import and therefore may be involved in adapting imported tRNAs to specific requirements of the mitochondrial translation machinery.

INTRODUCTION

For the majority of eukaryotes studied, mitochondrial tRNAs are encoded by the organellar genome. However, in a number of protozoa and higher plants, transport of nuclear-encoded tRNAs into mitochondria has been implicated (1, 2). These may either represent a subset of the total organellar tRNAs, as in higher plants (3), or they may represent the entire set, as in the protozoans *Trypanosoma brucei* (4) and *Leishmania tarentolae* (5). tRNA import has also been postulated in *S.cerevisiae* (6) even though the yeast organellar genome appears to encode all tRNAs necessary for protein synthesis (7). In that case, hybridization data suggest that a single nuclear-encoded $tRNA^{Lys}$ is imported into mitochondria. The function of the imported $tRNA^{Lys}$, however, is unclear as it cannot be aminoacylated by the mitochondrial charging enzyme (6).

Previous evidence for mitochondrial tRNA import was based primarily on indirect data such as the hybridization of organellar tRNAs to nuclear but not to mitochondrial DNA, and on the apparent absence of a complete set of tRNA genes from mitochondrial genomes whose sequences are known. The discovery of RNA editing in both plant and trypanosomal mitochondria (8) has since raised the alternative explanation that tRNAs may be created by extensive modification of transcripts from cryptic mitochondrial genes. Recently, direct *in vivo* evidence for mitochondrial tRNA import has been presented in three systems. A heterologous $tRNA^{Leu}$ expressed in transgenic potatoes was shown to be transported across mitochondrial membranes (9), and in *T.brucei* transfected with a mutated allele of its $tRNA^{Tyr}$ gene, *in vivo* import of the mutant gene product was demonstrated (10). Similar experiments have been performed independently in *L.tarentolae* using a plasmid based expression system and mutated versions of $tRNA^{Thr}$ as substrates (11). The nuclear origin of the imported tRNAs was clearly established in all cases as the genes encoding them were introduced by nuclear transformation.

In plants and in some protozoa, presumed nuclear-encoded mitochondrial tRNAs appear identical in primary sequence to their cytosolic counterparts (3). In trypanosomes, in which the entire set of organellar tRNAs is postulated to be imported, mitochondrion- and cytosol-specific forms have also been shown (4). These additional tRNAs may be required to account for differences in the genetic code between the nuclear and organellar genomes. Of the tRNAs that are present in both mitochondrial and cytosolic fractions, three homologous plant $tRNAs^{Leu}$ have been analysed in detail. Those nuclear-encoded tRNAs have a methylated guanosine at position 18 when they are present in the mitochondria; whereas this position is not methylated in the corresponding cytosolic forms (3, 12). It has been hypothesized that this modification may act as a signal for transport of the tRNAs across the mitochondrial membranes (2).

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We have compared the primary sequences of three nuclear-encoded mitochondrial tRNAs [$tRNA^{Lys}$(CUU), $tRNA^{Leu}$(CAA), and $tRNA^{Tyr}$(GUA)] with their cytosolic counterparts in *T.brucei* by using a combination of specific 3'-end splint labeling (13) and direct enzymatic sequence analysis. For each tRNA it was found that the primary sequences were identical in both fractions and, for $tRNA^{Tyr}$ and $tRNA^{Lys}$, were in agreement with the sequences predicted by the corresponding genes. However, for each of the tRNAs, a gap or distortion in the alkaline hydrolysis ladder, indicative of a nucleotide modification, was identified in the mitochondrial sample. The modification mapped to a conserved cytidine residue at position 32 at the penultimate position 5' of the anticodon. A requirement of the modified cytidine for mitochondrial tRNA import can be excluded because unspliced mutant $tRNA^{Tyr}$, purified from the organellar fraction of transformed cells, was not modified at that position. That result suggests that the modification performs an intramitochondrial function, such as in modulating the decoding properties of the corresponding anticodons.

MATERIAL AND METHODS

Strains

The wild type strain used was procyclic form IsTat 1.1 *T.brucei*. T.b.Mod and T.b.Sup were previously derived from that strain by stable transformation using the plasmids pHyg-Mod and pHyg-Sup respectively as transfecting DNA (21). T.b.Mod contains three point mutations in the anticodon stem-loop of one of the two $tRNA^{Tyr}$ gene alleles; two of these mutations are in the intron region and the third is a G to A transition at position 37, immediately 3' of the anticodon. These mutations were introduced from pHyg-Mod by homologous recombination. T.b.Sup contains the three point mutations plus a fourth mutation which is a G to C transversion at the 5' position of the anticodon. The presence of this fourth mutation interferes with splicing of the $tRNA^{Tyr}$ intron (21).

Isolation of mitochondria

Mitoplast and cytosolic fractions were prepared from 6 liters ($1-2\times10^7$ cells/ml) each of either the wild type or the transformed cell line. Cells were lysed under hypotonic conditions by passage through a hypodermic needle (26 gauge) (14) and centrifuged at 6000×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 and digested with DNAse I (final concentration 10 mg/ml) and micrococcal nuclease (final concentration 50 units/ml). After an additional centrifugation at 6000×g, the pellet was suspended in isotonic buffer containing 60% Percoll and fractionated on a 20–35% Percoll gradient (15).

3'-End splint labeling and sequencing of tRNAs

RNA was purified by the acid guanidinium isothiocyanate method as described elsewhere (16). For sequence analysis, tRNAs were specifically labeled at the 3'-terminus using the splint-labeling technique described by Hausner *et al.* (13). Identical amounts of cytosolic and mitochondrial RNA (5 μg each to label $tRNA^{Lys}$ and $tRNA^{Tyr}$, 20 μg each to label $tRNA^{Leu}$ and the unspliced mutant form of $tRNA^{Tyr}$) were incubated with 20 pmol of the corresponding oligonucleotide, either 100 μCi of [α-^{32}P]dCTP for labeling $tRNA^{Lys}$ and $tRNA^{Tyr}$ or 200 μCi for labeling $tRNA^{Leu}$ and unspliced mutant $tRNA^{Tyr}$, and 10 units of Sequenase (United tates Biochemical Corp.) in 20 μl of 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM $MgCl_2$ and 1 mM dithiothreitol. Reactions were incubated for 30 min at 37°C. The sequence of the oligonucleotides used to label each tRNA species were as follows: $tRNA^{Lys}$, 5'-GTGGCACCCCCGTGGGGCTCGAACCCA-3'; $tRNA^{Leu}$, 5'-GTGGTGACAAGAGTGGG-3'; $tRNA^{Tyr}$ and unspliced mutant $tRNA^{Tyr}$, 5'-GTGGTCCTTCCGGCCGGAATCGA-A-3'. Each of the oligonucleotides hybridize to the 3'-ends of the corresponding 3'-processed, CCA-containing tRNA and contains a protruding 5'-G residue. Incorporation of [α-^{32}P] dCTP opposite the G residues results in the specific labeling of 3'-processed tRNA molecules. These labeled RNAs were resolved on 8 M urea, 10% polyacrylamide gels; visualized by autoradiography; excised from the gel and recovered by elution into 0.5 M ammonium acetate (pH 8.0), 0.1% SDS, 0.1 mM EDTA and 20% phenol.

RNA sequencing reactions were prepared using the United States Biochemical Corp. RNA sequencing kit according to the manufacturer's instructions, except for the alkaline hydrolysis reactions, which were incubated at 95°C for 3 min. RNA sequencing reactions were resolved by electrophoresis on 8 M urea, 10% polyacrylamide gels.

RESULTS

The majority of mitochondrial tRNAs of *T.brucei* and their cytosolic counterparts are identical in terms of their electrophoretic mobilities on two-dimensional polyacrylamide gels (4). In this study we undertook a more detailed analysis of three specific tRNAs to determine if subtle differences may exist between their cytosolic and organellar forms. It has been suggested that such differences may be involved in either transport of the tRNAs into the mitochondria or in adaptation of the tRNAs for use by the two distinct translation systems. The three tRNAs studied were $tRNA^{Lys}$, $tRNA^{Tyr}$ and a $tRNA^{Leu}$ species. The primary sequences of the cytosolic and mitochondrial forms of these RNAs were examined after the tRNAs, present in purified subcellular fractions, were 3'-end labeled. Specific labeling of the individual tRNAs in either mitochondrial or cytoplasmic RNA fractions was achieved by 3'-end splint labeling using specific oligonucleotides and Sequenase as first described by Hausner *et al* (13). As a consequence of the posttranscriptional addition of CCA residues, the exact 3' sequences of mature tRNAs are known and they are therefore perfect substrates for the splint labeling protocol. This novel approach to determine the sequence of tRNAs was essential to these experiments as it allowed us to work with complex mixtures and limited amounts of material. The labeled tRNAs are purified by polyacrylamide gel electrophoresis; individual bands are identified by autoradiography, excised from the gel and the eluted RNA subjected to direct sequence analysis. Because enzymatic sequencing of RNAs can yield ambiguous results, especially with heavily modified substrates such as tRNAs, it is not by itself a sufficient tool to characterize novel tRNAs. However, the technique can yield important information in comparisons of the primary structures of tRNAs. In this study, direct sequence determinations of individual tRNAs were used (a) to compare the sequence patterns and potential nucleotide modifications of cytosolic and mitochondrial forms of individually labeled tRNA species, (b) to align the obtained primary structures to the sequences predicted from the corresponding genes, and (c) to

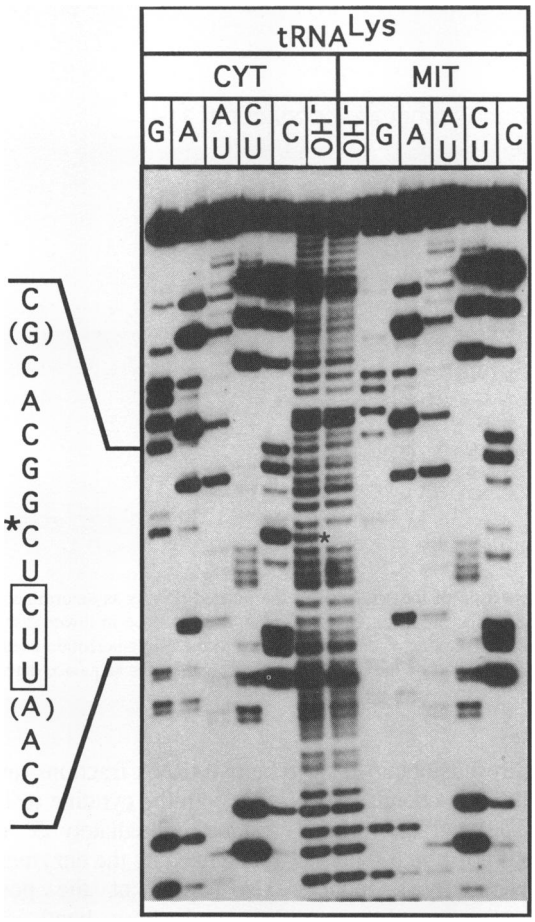

Figure 1. Cytosolic and mitochondrial tRNAs^{Lys} differ by a nucleotide modification. Cytosolic and mitoplast fractions of *T.brucei* tRNA^{Lys} were specifically labeled using the splint technique, purified by gel electrophoresis, and subjected to partial digestion with base-specific RNases and alkaline hydrolysis. RNase digests and the alkaline hydrolysis reaction were resolved by electrophoresis on an 8M urea, 8% polyacrylamide sequencing gel. The sequence indicated on the left corresponds to the predicted sequence for tRNA^{Lys}; nucleotides in parentheses could not be read and are inferred from the known gene sequence (17). The boxed nucleotides indicate the anticodon; the asterisk indicates the position of the nucleotide modification. CYT, cytosolic fraction; MIT, mitoplast fraction, G, RNase T1; A, RNase U2; A/U, RNase PhyM; C/U, RNase *Bacillus cereus*; C, RNase CL3.

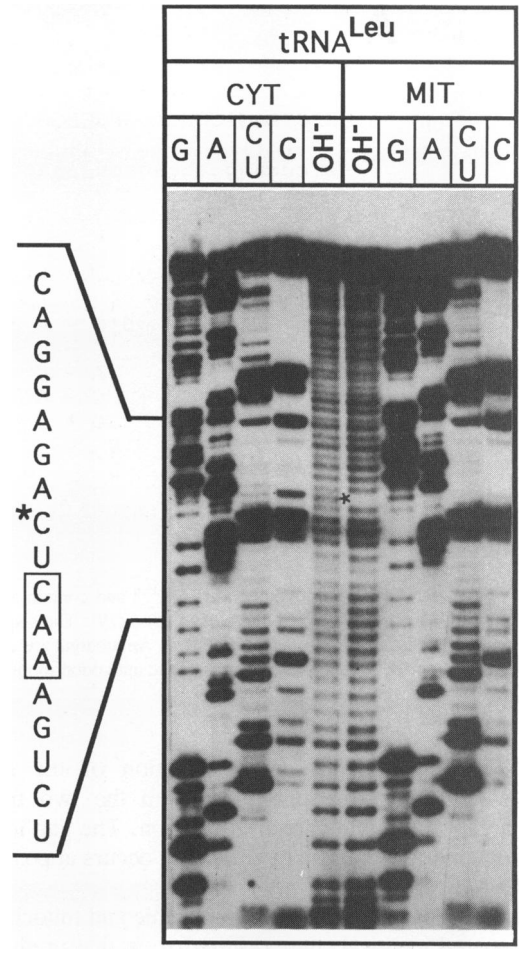

Figure 2. Cytosolic and mitochondrial tRNAs^{Leu} differ by a nucleotide modification. Cytosolic and mitoplast RNA fractions of *T.brucei* were hybridized to an oligonucleotide complementary to a tRNA^{Leu} species, labeled using the splint technique, purified by gel electrophoresis, and subjected to partial digestion with base-specific RNases and to alkaline hydrolysis. The digests were resolved by electrophoresis on an 8M urea, 10% polyacrylamide sequencing gel. The sequence indicated by the reactions is shown on the left; the sequence shares identity with that predicted for the tRNA^{Leu} whose gene has been cloned (see Fig. 3). The predicted anticodon is boxed; the asterisk indicates the position of the nucleotide modification. CYT, cytosolic fraction; MIT, mitoplast fraction; G, RNase T1; A, RNase U2; C/U, RNase *B.cereus*; C, RNase CL3.

position the anticodon and potential mitochondrial or cytosolic specific modifications within the tRNA molecules.

tRNA^{Lys} was chosen first for study because all four members of the *T.brucei* tRNA^{Lys} gene family have been cloned, sequenced, and shown to have almost identical coding regions (17). In addition, Northern analysis indicates the presence of tRNA^{Lys} in both cytosolic and mitochondrial fractions. tRNA^{Lys} in the two subcellular fractions was splint labeled using an oligonucleotide complementary to its 3′ end and the labeled molecules were subjected to RNA sequencing. Identical sequence patterns for the cytosolic and mitochondrial fractions were obtained using the base-specific ribonucleases T_1, U_2, PhyM, *B.cereus*, and CL_3 (Fig. 1). Although the RNase digestion results appeared identical between the two RNA fractions, a comparison of the alkaline hydrolysis ladders revealed a gap in the mitochondrial sample. The presence of this gap is indicative of a nucleotide modification which interferes with hydrolysis of the phosphate-ribose backbone of the RNA. The location of the gap indicates that the modification is on the cytidine residue which is nucleotide 32 in the tRNA. This uridine is located at the penultimate position 5′ of the anticodon and is conserved in the majority tRNAs that have been sequenced (18).

To investigate whether the nucleotide modification detected in tRNA^{Lys} is present in other mitochondrial tRNAs, the sequence analysis was extended to tRNA^{Leu}. All 6 possible leucine codons are used in *T.brucei* and a Southern analysis indicated that there are at least 6 homologous tRNA^{Leu} genes encoded in the nuclear genome. One of these 6 genes has been sequenced (19) and an oligonucleotide which is complementary to the predicted 3′ end of the encoded tRNA was synthesized. Splint labeling of mitochondrial and cytosolic RNAs was performed using this oligonucleotide and the labeled molecules were gel-purified. As with tRNA^{Lys}, identical sequence patterns were obtained for the cytoplasmic and mitochondrial fractions using base-specific

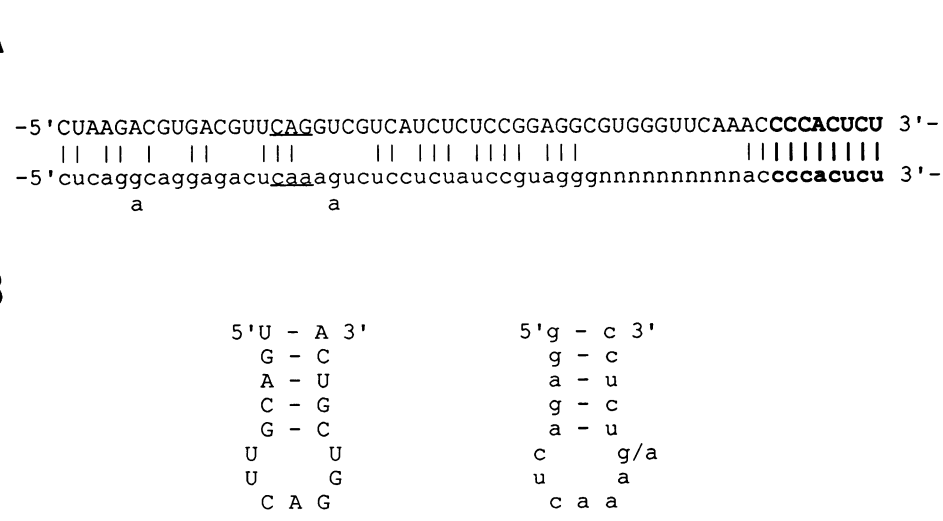

Figure 3. Labeled tRNAs are homologous to $tRNA^{Leu}$ and contain a CAA anticodon. **A**, comparison of the sequence of the labeled tRNAs as determine in Fig. 2 with that predicted for a $tRNA^{Leu}$ by the cloned gene (19). The sequence in upper case letters is that predicted from the gene; the sequence in lower case letters is that determined for the splint-labeled $tRNA(s)^{Leu}$. Anticodons are underlined; bold letters indicate regions of complementarity to the oligonucleotide used in the splint-labeling reaction. **B**, secondary structures of the anticodon loops of the $tRNA^{Leu}$ species as predicted by the cloned gene (left) and by the sequence determined in Fig. 2 (right).

RNAses (Fig. 2). Again, an examination of the alkaline hydrolysis ladders of the $tRNAs^{Leu}$ from the two fractions revealed a gap in the mitochondrial fraction. The gap indicates that a mitochondrion-specific modification occurs at position 32 in the tRNA.

Although the sequences of the cytoplasmic and mitochondrial RNAs appeared identical, they differed from that predicted by the cloned $tRNA^{Leu}$ gene (19) (Fig. 3). The most likely interpretation of this result is that the tRNA encoded by the cloned gene is not highly expressed and another, more abundant $tRNA^{Leu}$ having a highly homologous 3′ end was the main splint labeled species. The sequenced $tRNA^{Leu}$ most likely comigrates with the $tRNA^{Leu}$ encoded by the cloned gene as only one band was obtained upon splint labeling. Comparison of the two tRNAs reveals that they have different anticodons but are otherwise fairly homologous. The two sequences show 31 identities over 47 nucleotides that can be read, giving a predicted homology of 66% (Fig. 3A). The position of the anticodon in the sequence of the labeled $tRNA^{Leu}$ is defined by compensatory base changes which conserve the structure of the anticodon stem (Fig. 3B). Using this information, it is possible to map the mitochondrial-specific modification to the identical position as that found in $tRNA^{Lys}$, to the cytidine residue 5′ of the anticodon.

The $tRNA^{Tyr}$, whose gene is interrupted by an intron (20, 21), was chosen as a third tRNA to be sequenced because it is encoded by a single copy gene (17). In addition, we have previously reported its maturation pathway (21) and provided direct evidence for its import to mitochondria (10). The sequences of both cytosolic and organellar forms of the tRNA are in agreement with that predicted for the mature $tRNA^{Tyr}$ by the gene sequence (Fig. 4). As with $tRNA^{Lys}$ and $tRNA^{Leu}$, the alkaline hydrolysis ladder provides evidence for specific modification of the mitochondrial sample. However, the pattern observed with $tRNA^{Tyr}$ is more complex than that observed with the previous tRNAs and suggests that mitochondrial $tRNA^{Tyr}$ is specifically modified at two adjacent nucleotides. A comparison of the RNase digestion results obtained with the two tRNA fractions revealed that, in the mitochondrial $tRNA^{Tyr}$, both the cytidine residue at position 32 and the uridine residue immediately 5′ of the anticodon (position 33) are not recognized by the enzymes. The alkaline hydrolysis ladders also differ at the positions corresponding to these residues and those bands in the mitochondrial sample which represent cleavages 5′ of the residues are displaced in a manner that suggests that one or both of the modifications is an addition of a bulky residue. These results suggest that the cytidine and uridine residues are either specifically modified in the *T.brucei* mitochondrion or in the cytoplasm immediately prior to or during their import.

Previously, we have described the construction of *T.brucei* strains which express mutant forms of the $tRNA^{Tyr}$ (21). In the transformed cell line T.b.Mod, one allele of the $tRNA^{Tyr}$ gene has been replaced by a mutated version which expresses $tRNA^{Tyr}$ carrying three point mutations, two in the intron region and one which is a G to A transition of the residue immediately 3′ of the anticodon. It has been shown that the mutant RNA is spliced with an efficiency comparable to that of wild type (21). To determine whether the mutations present in that molecule affect the mitochondrion-specific modification of $tRNA^{Tyr}$, we prepared mitoplast and cytosolic RNA fractions from wild-type *T.brucei* and from the mutant strain T.b.Mod. $tRNAs^{Tyr}$ from these fractions were 3′-end labeled and subjected to RNA sequence analysis (Fig. 5). The alkaline hydrolysis ladder observed with the tRNAs originating from the T.b.Mod strain differed from that obtained with the wild type strain in that only one gap at position 32, instead of two adjacent gaps (positions 32 and 33), was observed. The hydrolysis pattern obtained with the mutant strain was therefore very similar to those obtained with $tRNA^{Lys}$ and $tRNA^{Leu}$, that is, a single gap indicative of a modification of the conserved cytidine was present. Approximately 50% of the molecules seem to be unmodified at position 33 as is expected if both the wild type and the mutant alleles are transcribed. These results suggest that the mutations

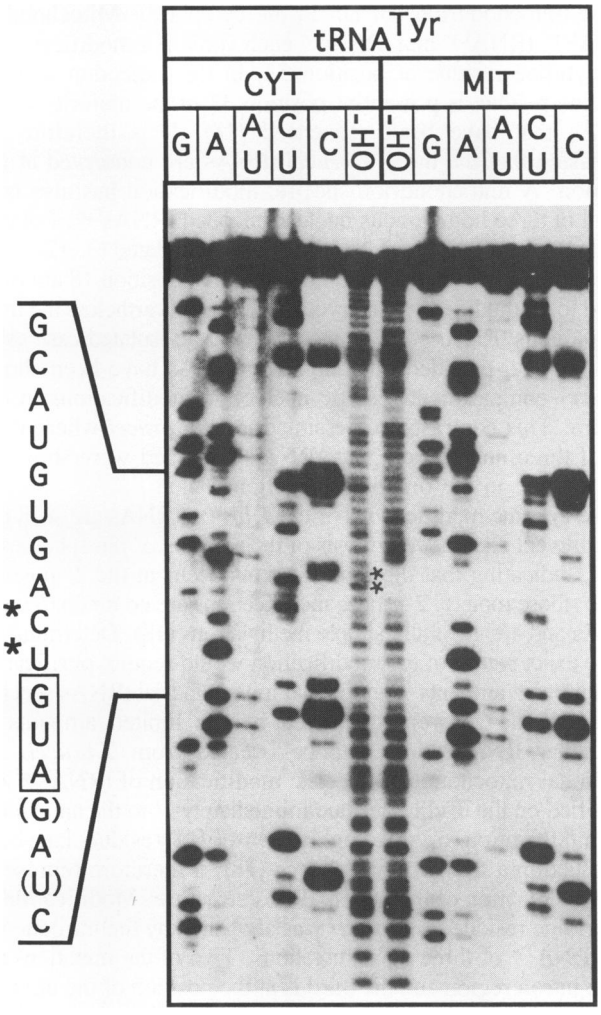

Figure 4. Cytoplasmic and mitochondrial $tRNA^{Tyr}$ differ by two nucleotide modifications. Mitoplast and cytosolic fractions of *T.brucei* $tRNA^{Tyr}$ were specifically labeled using the splint technique, purified by gel electrophoresis, and subjected to partial digestion with base-specific RNases and to alkaline hydrolysis. The digests and the alkaline hydrolysis reactions were resolved by electrophoresis on an 8M urea, 8% polyacrylamide sequencing gel. The sequence indicated on the left corresponds to the predicted sequence for $tRNA^{Tyr}$; nucleotides in parentheses could not be read and are inferred from the known gene sequence (17). The boxed nucleotides indicate the anticodon; the asterisks indicate the positions of the nucleotide modifications. CYT, cytosolic fraction; MIT, mitoplast fraction; G, RNase T1; A, RNase U2; A/U, RNase PhyM; C/U, RNase *B.cereus*; C, RNase CL3.

present in the T.b.Mod strain affect recognition of $tRNA^{Tyr}$ by the enzyme(s) that modifies the uridine at position 33 but not by the enzyme that modifies the conserved cytidine at position 32. Furthermore, those data are consistent with the interpretation that the cytidine modification in $tRNA^{Tyr}$ is similar or identical to the cytidine modifications that were identified in the previous tRNAs.

The results described above indicate that the conserved cytidine residue in the anticodon loop is modified in all three mitochondrial tRNAs examined and suggest the hypothesis that a nucleotide modification might be present in the same relative position in all mitochondrial tRNAs. Because mitochondrial tRNAs in trypanosomes are encoded in the nucleus, they must be imported into the organelle. An attractive hypothesis is that the modification

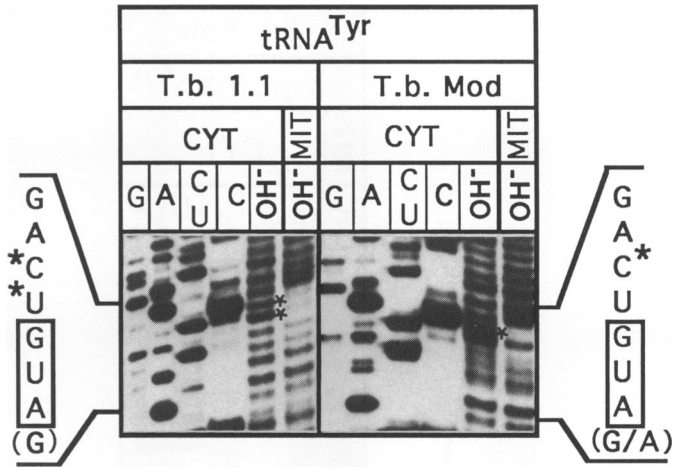

Figure 5. Introduction of mutations in $tRNA^{Tyr}$ prevents modification of the U residue (position 33) but not of the C residue at position 32. Cytosolic and mitoplast fractions of $tRNA^{Tyr}$ in both wild type *T.brucei* (T.b. 1.1) and in the transformed strain (T.b.Mod) (21) were specifically labeled, purified by gel electrophoresis, and subjected to partial digestion with base-specific RNases and to alkaline hydrolysis. The digests and the alkaline hydrolysis reactions were resolved by electrophoresis on an 8M urea, 8% polyacrylamide sequencing gel. The strain T.b.Mod expresses, in addition to the wild type $tRNA^{Tyr}$, a mutant form of $tRNA^{Tyr}$ carrying 3 mutations in the anticodon stem of the intron-containing precursor; one of these mutations (the G to A transition at the ultimate position 3′ of the anticodon) is retained in the mature tRNA (21). The sequences indicated correspond to those predicted for wild type and mutant $tRNA^{Tyr}$; nucleotides in parentheses could not be read and are inferred from the known gene sequence (17). The boxed nucleotides indicate the anticodon; asterisks indicate the positions of nucleotide modifications. CYT, cytosolic fraction; MIT, mitoplast fraction; G, RNase T1; A, RNase U2; C/U, RNase *B.cereus*; C, RNase CL3.

might act as a signal for recognition of the tRNAs by the mitochondrial transport system. Alternatively, the modification may be involved in an exclusively intramitochondrial function.

To test the former hypothesis, we made use of an *in vivo* import system, by which evidence for mitochondrial import of an *in vitro* mutagenized $tRNA^{Tyr}$ has been demonstrated (10). The strain T.b.Sup expresses a $tRNA^{Tyr}$ which contains the same three mutations as are present in the mutant T.b.Mod tRNA plus a fourth mutation of the 5′ nucleotide of the anticodon. The mutations were originally introduced to tag the molecule and lead to accumulation, both in the cytosol and in the mitochondrion, of the unspliced $tRNA^{Tyr}$ precursor (10). If the cytidine modification is required for transport of tRNAs, then it should be present in the organellar fraction of unspliced $tRNA^{Tyr}$. RNAs purified from the cytosolic and mitochondrial fractions of the transformed cell line were splint labeled using the same oligonucleotide, which was also used to label the mature wild type tRNA. Labeled RNAs corresponding in size to unspliced $tRNA^{Tyr}$ were gel-purified and subjected to sequence analysis. As expected, the analysis revealed the presence of the intron and the introduced mutations (Fig. 6). Contrary to the results obtained with mature wild type $tRNA^{Tyr}$, however, the alkaline hydrolysis ladders of the two precursor fractions were identical; there is no evidence for mitochondrion-specific modifications. The mutated unspliced $tRNA^{Tyr}$ is therefore not a substrate for the modification enzymes, most likely as a result of the presence of the intron, which occurs 5 nucleotides 3′ of the conserved cytidine. Alternatively, the introduction of the 4 mutations may interfere with recognition of the tRNA by the modification

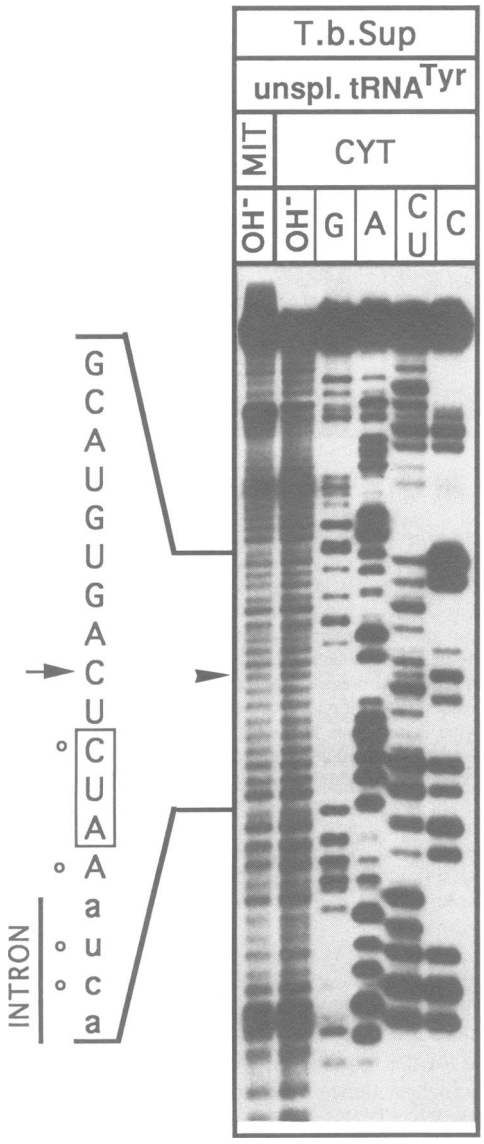

Figure 6. The conserved cytidine is not modified in unspliced, mitochondrial $tRNA^{Tyr}$. Cytosolic and mitoplast fractions of $tRNA^{Tyr}$ from the transformed strain T.b.Sup (21) were labeled using the splint technique. The strain T.b.Sup carries 4 mutations in one of the two $tRNA^{Tyr}$ gene alleles; these mutations cause the accumulation, both in the mitochondria and in the cytoplasm, of the unspliced precursor (10). The labeled precursor tRNAs were gel-purified and subjected to alkaline hydrolysis only (mitoplast fraction) or to alkaline hydrolysis and base-specific RNases (cytosolic fraction). The digests were resolved by electrophoresis on an 8M urea, 8% polyacrylamide sequencing gel. The sequence shown on the left corresponds to that of unspliced, mutant $tRNA^{Tyr}$. The boxed nucleotides indicate the anticodon; circles mark the positions of the 4 nucleotide mutations. Nucleotides written in lower case are part of the intron; the arrow indicates the position of the cytidine residue (position 32) which is modified in the mature RNA. MIT, mitoplast fraction; CYT, cytosolic fraction; G, RNase T1; A, RNase U2, C/U, RNase *B.cereus*; C, RNase CL3.

enzymes. The data indicate that a modified cytidine residue at position 32 in the tRNA is not required for its import into the trypanosomal mitochondrion.

DISCUSSION

RNA sequence analysis of three nuclear-encoded tRNAs in *T.brucei* indicates that they are specifically modified when present in the mitochondrion but not in the cytoplasm. Mitochondrial $tRNA^{Leu}$, $tRNA^{Lys}$ and $tRNA^{Tyr}$ each contain a modification of the cytidine residue at position 32, in the anticodon loop. A cytidine residue is present at position 32 in the majority of the tRNAs that have been sequenced (18). It is therefore not surprising that the three *T.brucei* tRNAs are conserved at this position. A mitochondrion-specific modification has also been found in three homologous nuclear-encoded $tRNAs^{Leu}$ of plants, in which the guanosine at position 18 is methylated (3, 12). Both the cytidine at position 32 and nucleotides at position 18 are often found to be modified in eukaryotic tRNAs. Nevertheless the three homologous $tRNAs^{Leu}$ in plants represent an isolated case as no other nuclear-encoded mitochondrial tRNAs have been shown to carry compartment-specific nucleotide modifications in this system. This contrasts to the situation in *T.brucei* where three out of three non-homologous tRNAs examined were shown to be modified in the organellar fraction.

The cytidine modifications of the *T.brucei* tRNAs are such that they prevent alkaline hydrolysis of the adjacent 3′ phosphodiester bond, indicating that the modifications occur at the 2′ position of the ribose moiety. 2′-ribose modifications are common to many tRNAs and are generally simple methylations (18). Determination of the exact nature of the modification would require purification of significant amounts of individual mitochondrial tRNAs species. Efforts to do so were hampered by the limited amounts of organellar RNAs which can be isolated from *T.brucei*. An additional mitochondrion-specific modification of $tRNA^{Tyr}$ was identified on the uridine residue immediately 5′ to the anticodon. No modification of this highly conserved residue has been described in a tRNA of any source (18); it therefore represents a novel deviation of the classic tRNA structure. Modification of the uridine residue in *T.brucei* was abolished by the introduction into $tRNA^{Tyr}$ of three point mutations. Two of the mutations are in the intron region and the third is at the position of the ultimate nucleotide 3′ of the anticodon. As very little intron-containing precursor $tRNA^{Tyr}$ is present in wild-type *T.brucei*, we have not been able to determine if the modification of the uridine residue occurs prior or subsequent to splicing. If it occurs subsequent to the splicing reaction, during which the two intron mutations are removed, then the single G to A transition of the nucleotide immediately 3′ of the anticodon is responsible for preventing recognition of the tRNA by the modification enzyme. Because the uridine modification is specific to the $tRNA^{Tyr}$, it is not surprising that it is affected by minor changes in the primary sequence. On the other hand, the mutations were not expected to affect modification of the conserved citydine residue at position 32 as it occurs in several, non-homologous tRNAs. If the cytidine modifications in these multiple tRNAs are identical, then it is likely that the modifying enzyme(s) recognizes some aspect of secondary structure rather than the primary sequence of the RNAs.

The abundance of nucleotide modifications both in the cytoplasmic and in the mitochondrial tRNAs, resulted in occasional gaps or ambiguities in our sequence analyses that were not resolved. However, the majority of the nucleotides could be identified and no differences were detected between the primary sequences of the mitochondrial and cytoplasmic tRNA fractions. In addition, the primary sequences of $tRNA^{Lys}$ and $tRNA^{Tyr}$ isolated from either the cytosol or the mitochondria were in agreement with those predicted from their respective genes. The cytoplasmic and mitochondrial $tRNAs^{Leu}$ were also identical in primary sequence, although the sequence differed from that of

the cloned $tRNA^{Leu}$ gene. These tRNAs are most likely the transcripts of another member of the $tRNA^{Leu}$ gene family which has not yet been cloned. Taken together, these data provide no evidence for editing of trypanosome tRNAs. This result is in contrast to the extensive editing of mitochondrial mRNAs which has been observed in *T.brucei* (8) and to the recently reported editing of tRNAs in *Acanthamoeba castellanii* (22), marsupial (23) and plant mitochondria (24). Although our data does not exclude the possibility that tRNAs other than $tRNAs^{Leu, Lys, Tyr}$ are substrates for RNA editing, it indicates that if tRNA editing does occur in trypanosomes, it is not the exclusive mechanism for the production of organellar tRNAs.

Direct evidence for mitochondrial RNA import has now been provided in plants (9), trypanosomes (10) and *Leishmania* (11). In two of these systems, the only difference found between mature cytosolic tRNAs and their organellar counterparts has been the presence of unique nucleotide modifications in the mitochondrial molecules. It has therefore been hypothesized, assuming the modification of the tRNAs occurs in the cytoplasm, that the modified nucleotides provide a signal for mitochondrial import. In trypanosomes, we have shown that the cytidine modification is not required for import as it was not detected in an *in vivo* expressed artificially altered $tRNA^{Tyr}$, whose import has clearly been established. The altered $tRNA^{Tyr}$ accumulates in its unspliced, unmodified form in both cytosol and mitochondria (10). An alternative hypothesis for the import of tRNAs in trypanosomes suggests that the recognition signals may be present in high molecular weight forms of the RNAs rather than in the mature forms. Such high molecular weight tRNA forms have been detected in trypanosome mitochondria by Hancock *et al.* (25) and by Schneider *et al.* (10). Future research concerning import signals will therefore focus on these presumably unprocessed molecules.

As the modified cytidine is not required for import, it most likely performs an intramitochondrial function, such as in the adaptation of tRNAs for use by the organellar translation machinery. Lustig *et al.* (26) have recently shown that the nucleotide at position 32 of *Escherichia coli* $tRNA^{Gly}$ decisively influences the reading properties of the anticodon. One hypothesis that is suggested by the observation is that the nucleotide at position 32 affects the interaction between tRNA and the 16S rRNA. Previous experiments indicate that the anticodon stem-loop extremity of tRNA interacts with 16S rRNA at positions that are located in the cleft of the 30S ribosome subunit (reviewed in 27). In particular, the wobble base (position 34) of certain *E.coli* tRNA−ribosome complexes is very closely juxtaposed to a highly-conserved sequence in the 16S rRNA (28). Although this sequence is conserved in the cytoplasmic small subunit rRNA of *T.brucei*, the corresponding region of the mitochondrial small subunit rRNA is highly diverged (29). It is therefore intriguing to speculate that the cytidine modification at position 32 serves to promote the interactions between nuclear-encoded tRNAs in *T.brucei* and the organellar rRNA. Unfortunately, this hypothesis cannot be easily examined at the present because of the lack of a system of *in vitro* mitochondrial translation.

ACKNOWLEDGEMENTS

We thank Dr K.Watkins for his generous advice and the members of our laboratory for their helpful discussions. This work was supported by a grant from the John D. and Catherine T. MacArthur Foundation and by National Institutes of Health Grant AI21975. A.S. was supported by the Swiss National Foundation for Scientific Research Grant 823A-030686. N.A. is a Burroughs Wellcome Scholar of Molecular Parasitology.

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