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Cytosolic yeast $tRNA^{His}$ is covalently modified when imported into mitochondria of *Trypanosoma brucei*

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ABSTRACT

The mitochondrial genome of *Trypanosoma brucei* does not encode any tRNAs. Instead, mitochondrial tRNAs are synthesized in the nucleus and subsequently imported into mitochondria. The great majority of mitochondrial tRNAs have cytosolic counterparts showing identical primary sequences. The only difference found between mitochondrial and cytosolic isotypes of the tRNAs are mitochondria-specific nucleotide modifications which appear to be a common feature of imported tRNAs in trypanosomes. In this study, a mutated yeast cytosolic $tRNA^{His}$ was expressed in trypanosomes and its import phenotype was analyzed by cell fractionation and nuclease treatment of intact mitochondria. Furthermore, cytosolic and mitochondrial isotypes of the yeast $tRNA^{His}$ were specifically labeled and analyzed by limited alkaline hydrolysis. These experiments revealed the presence of mitochondria-specific nucleotide modifications in the yeast $tRNA^{His}$. The positions of the modifications were determined by direct enzymatic sequencing of the $tRNA^{His}$ and shown to correspond to the ultimate and penultimate nucleotides before the anticodon, the same relative positions which are modified in the mitochondrial isotype of trypanosomal $tRNA^{Tyr}$. The results demonstrate that covalent modification of tRNAs in trypanosomal mitochondria can be used, in analogy to processing of precursor proteins during mitochondrial protein import, as a marker for import of both endogenous and heterologous tRNAs.

INTRODUCTION

Whereas protein translocation across membranes has long been a major theme in cell and molecular biology (1), much less is known about transport of RNA across membranes (2). Since RNA is synthesized in the nucleus but almost exclusively used in the cytoplasm, it has to be exported. It has become clear recently that for some RNAs the cytosol is not the final destination, but that they are further transported across the mitochondrial membranes. This process is very different from nuclear RNA export since, unlike the nuclear membranes, the mitochondrial inner membrane does not have permanent pores and exhibits a membrane potential. Mitochondrial RNA import appears to be a universal process within eukaryotes. It has been shown that import of tRNAs plays a prominent role in the mitochondrial biogenesis of plant and protozoa (3–5).

The parasitic protozoan *Trypanosoma brucei* represents an especially good system to study mitochondrial tRNA import. Its mitochondrial genome lacks any identifiable tRNA genes, implying that the whole set of mitochondrial tRNAs is imported from the cytosol (6,7). Using an *in vivo* import system it was shown that in *T.brucei* tRNAs are imported into mitochondria independently of their genomic context or their genetic origin (8). The great majority of tRNAs have a dual location, in both the cytosol as well as in mitochondria. Only few compartment-specific tRNAs have been described (7). tRNAs imported into mitochondria may acquire covalent modifications. This was first shown for a number of $tRNAs^{Leu}$ in plants which are specifically methylated at the guanosine at position 18 when imported into mitochondria (9,10). In *T.brucei* it was demonstrated that a mitochondria-specific nucleotide modification concerning the conserved cytidine residue at position 32 most likely is a general feature of imported tRNAs. However, it was also shown that this modification represents a consequence rather than a signal for import since a mutated variant of a tRNA which cannot be modified anymore can still be imported into mitochondria (11).

The aim of this study was to demonstrate that this nucleotide modification, even though it is not causatively involved in tRNA import, can be used as marker for the mitochondrial localization of a heterologous tRNA expressed in *T.brucei*.

MATERIALS AND METHODS

Strains

Procyclic wild-type and transformed *T.brucei*, stock 427, were grown in SDM-79 medium supplemented with 5% fetal bovine serum. Electroporation and transfection of the cells were performed as described (12). The pTbo-1 derivatives, which due to presence of a minicircle sequence are maintained and replicated as episomes in *T.brucei*, were used as vectors to establish the transformed cell lines (13). Cell line Y-H25, which has been characterized before (8), was transfected with pY-H25 containing the gene for the yeast cytosolic $tRNA^{His}$ in the context of 25 bp upstream flanking region of the trypanosomal $tRNA^{Tyr}$ gene and 65 bp of its own 3′-flanking regions. The newly established cell line Y-H25C was obtained by transfection with a variant of pY-H25, called pY-H25C, whose $tRNA^{His}$ gene had been mutated by replacing the uridine at position 32 by a cytidine (Fig. 1B).

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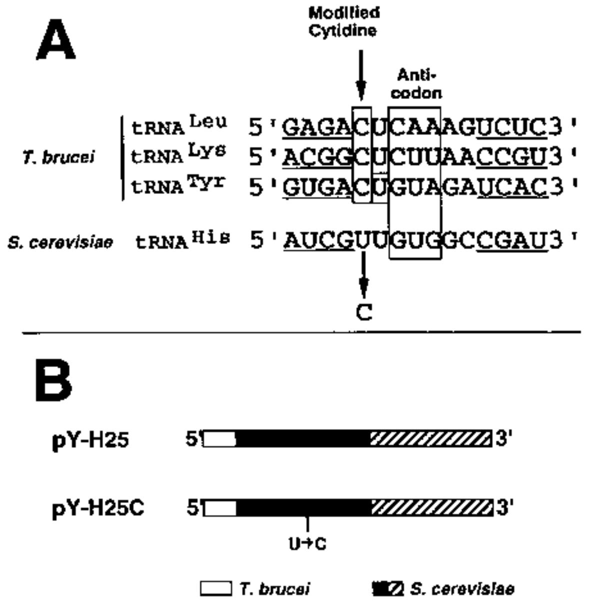

Figure 1. Mitochondria-specific nucleotide modifications in trypanosomal tRNAs. (**A**) Alignment of anticodon stem–loop sequences of three trypanosomal tRNAs and a yeast cytosolic tRNA^{His}. Complementary nucleotides building the anticodon stem are underlined. The anticodons are marked through the box on the right. The modified cytidine at the penultimate position before the anticodon which is common to the mitochondrial isotypes of all the trypanosomal tRNAs shown is indicated by the box on the left. The mitochondria-specific nucleotide modification exclusively found in the trypanosomal tRNA^{Tyr} is indicated through the boxed uridine before the anticodon. The U to C mutation introduced into the yeast tRNA^{His} is indicated. (**B**) Inserts of the plasmids used to transfect *T.brucei*. pY-H25 contains a yeast cytosolic tRNA^{His} gene flanked by 25 bp upstream sequence of the trypanosomal tRNA^{Tyr} gene and 65 bps of its own 3′-flanking sequence. pY-H25C contains the same insert as pY-H25 except that the uridine at position 32 has been replaced by a cytidine.

Isolation of cytosolic and mitochondrial RNA

Mitochondrial and cytosolic fractions were prepared from 6 l ($1–2.5 \times 10^7$ cells/ml) of the transformed cell lines. Cells were lysed under hypotonic conditions by passage through a hypodermic needle as described (14). Due to the hypotonic conditions which disrupt the mitochondrial outer membrane, mitochondria are converted into mitoplasts. An aliquot of the supernatant was used to prepare cytosolic RNA. The RNA was purified by the acid guanidinium isothiocyanate method as described (15).

3′-End splint labeling

All isolated RNA fractions were treated with RQ-DNase (Promega) prior to the labeling. For sequence analysis, tRNAs were specifically labeled at the 3′-terminus using the splint-labeling technique (16). Labeling of the trypanosomal tRNA^{Lys} was performed using the oligonucleotide 5′-GTGGCACCCCCGTGGGGCTCGAACC-CA-3′ (11). The oligonucleotide 5′-GGGTGGTGCCATCTCCTA-GAATCGAA-3′ was used as a splint to label the yeast tRNA^{His}, it hybridizes to the very 3′-end including the posttranscriptionally added CCA of yeast tRNA^{His} leaving a stretch of three Gs as a 5′-overhang. Under optimal conditions this oligonucleotide should allow addition of up to three radioactive cytidines to the 3′-end of the tRNA. The reaction conditions for the yeast tRNA^{His} were as follows: 20 µg DNase-treated cytosolic and mitochondrial RNAs isolated from the cell line transformed with the mutated yeast tRNA^{His} was incubated with 20 pmol of the oligonucleotide, 200 µCi of [α-^{32}P]dCTP (6000 Ci/mmol), 10 U Sequenase (United States Biochemical Corp.) in 10 µl 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. In order to extend the molecules containing only one or two radioactive cytidines the reaction was chased by adding unlabeled dCTP to 100 µM and further incubation for 5 min at identical temperature. Subsequently the samples were precipitated by isopropanol washed with 80% ethanol and resolved on 8 M urea, 10% polyacrylamide gels. Labeled bands were visualized by autoradiography on the wet gel, excised and the tRNA was recovered by elution into 0.5 M ammonium acetate (pH 8.0), 0.1% SDS, 0.1 mM EDTA and 20% phenol.

Limited alkaline hydrolysis and RNA sequencing

Limited alkaline hydrolysis of cytosolic and mitochondrial samples of labeled trypanosomal tRNA^{Lys} and yeast tRNA^{His} was achieved by incubation in 5 µl 50 mM Na-carbonate (pH 9.5) for 6 min at 90°C. After addition of 5 µl of 8 M urea containing 0.04% bromphenolblue the sample was immediately analyzed on a 8 M urea, 10% polyacrylamide gel.

The splint-labeled cytosolic yeast tRNA^{His} expressed in *T.brucei* was sequenced using the base-specific RNases T1, U2, *Bacillus cereus* and PhyM (Pharmacia). Gel-eluted labeled tRNA^{His}, 20 000 c.p.m. each, was used in each reaction. Sequencing reactions were performed in the presence of 3 µg carrier yeast tRNA for 15 min at 50°C using 1 U each of the enzymes. RNase T_1 and U_2 digestions were performed in 10 µl 25 mM Na-citrate (pH 3.5) containing 7 M urea and 0.04% bromphenolblue. RNase PhyM treatment was done in 10µl of the same buffer which had been adjusted to pH 5.0. *Bacillus cereus* RNase digestion was performed in 5 µl 50 mM Na-citrate (pH 5.0) containing 1 mM EDTA, after completion of the reaction 5 µl 10 M urea supplemented with 0.04% bromphenolblue was added. After incubation all samples were quenched on ice and immediately resolved on a 8 M urea, 10% polyacrylamide gel.

Miscellaneous

Northern blots and micrococcus nuclease digestion were performed as described (8).

RESULTS

The sequences of the anticodon stem loops of trypanosomal tRNA^{Lys}, tRNA^{Leu} and tRNA^{Tyr} (11) are compared with the corresponding sequence of cytosolic tRNA^{His} of *Saccharomyces cerevisiae* (17) (Fig. 1A). All trypanosomal tRNAs carry a mitochondria-specific cytidine modification at position 32. The tRNA^{Tyr} contained in addition a second adjacent nucleotide modification (uridine 33) which was also exclusively found in mitochondria. The exact nature of the nucleotide modifications are unknown, the most common one found at position 32 in other organisms is a methylation. The uridine at position 33 is not modified in any other species. In *T.brucei* it most likely consists of a bulky group resulting not only in a gap but also in a compression of the alkaline hydrolysis ladder (11). The yeast

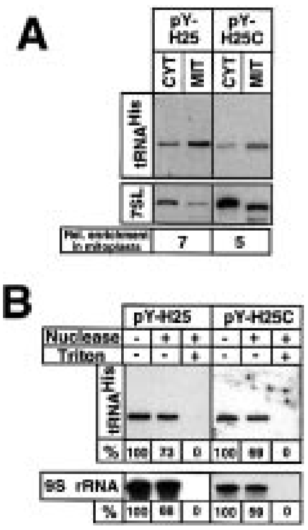

Figure 2. Wild-type and mutant yeast cytosolic tRNA^{His} are imported into mitochondria. (**A**) Northern blot containing 0.8 µg cytosolic (CYT) and 4 µg mitoplast (MIT) RNA from cells transfected with either pY-H25 or pY-H25C was hybridized with the same oligonucleotide which was used to splint label the yeast tRNA^{His} (upper) (see Materials and Methods). Subsequently the blot was reprobed with an oligonucleotide recognizing cytosolic 7SL RNA (lower) (8). The relative enrichment of the tRNAs^{His} in mitoplasts was determined by comparing the ratios of the tRNA^{His} and 7SL RNA signals in both the cytosolic and the mitoplast fractions. (**B**) Mitoplasts originating from the tRNAs^{His}-expressing transformants were either left untreated or were incubated with micrococcal nuclease (Nuclease) in the presence or absence of 1% Triton X-100 (Triton) and their respective RNAs were separated on a polyacrylamide gel. The corresponding Northern blot was probed for yeast tRNAs^{His} (upper) and subsequently reprobed for mitochondrial 9S rRNA (lower). The percentages of nuclease-resistant molecules, when compared with untreated samples, are indicated at the bottom of each panel.

tRNA^{His} unlike the three trypanosomal tRNAs contains a uridine at position 32. To increase chance that the yeast tRNA becomes modified when present in trypanosomal mitochondria, the nucleotide encoding the uridine at position 32 was changed by site directed mutagenesis to the conserved cytidine found in most other tRNAs (18).

Transfection of trypanosomes with the corresponding plasmid yielded cell line Y-H25C expressing the mutated tRNA^{His} (Fig. 1B). *In vivo* import into mitochondria of the mutated tRNA^{His} was compared with the wild-type yeast tRNA^{His}, expressed in the cell line Y-H25, which had been analyzed before (8). Mitochondrial import of both yeast tRNAs^{His} was analyzed by cell fractionation and subsequent Northern hybridizations using specific oligonucleotides as probes. Figure 2A shows that the wild-type and the modified yeast tRNA co-fractionate with the cytosol as well as with mitoplasts (see Materials and Methods) and are found to be enriched in the organelles to similar extents. In addition both molecules are resistant to externally added nuclease in intact mitoplasts and are only degraded after destroying the membranes by detergents (Fig. 2B). It is therefore concluded that the uridine at position 32 of the tRNA^{His} can be replaced by a cytidine without any effect on import.

For further analysis the yeast tRNA^{His} present in the cytosolic and mitochondrial fractions of the cell line Y-H25C was specifically labeled by the 3′-splint labeling method (16). This method allows highly efficient addition of radioactive [α-^{32}P]dCTP to the 3′-end of distinct tRNAs using specific oligonucleotides and Sequenase (see Materials and Methods). The specificity of the labeling was demonstrated by performing the reactions with total RNA either isolated from cell lines transformed with the plasmid only (Fig. 3A, pTbo) or with the plasmid containing the mutated yeast tRNA^{His} gene (Fig. 3A, pY-H25C). A signal is only detected in the cell line expressing the yeast tRNA^{His} but not in the control. For further analysis, labeled cytosolic and mitochondrial isotype of the tRNA^{His} were separated on a preparative sequencing gel; individual bands were identified by autoradiography, excised and eluted. The eluted 3′-labeled tRNAs were then subjected to partial alkaline hydrolysis. This technique can be used to detect nucleotide modifications by means of their interference with the alkaline hydrolysis reaction resulting in gaps and/or compressions in the hydrolysis ladder. Alkaline hydrolysis profiles of mitochondrial and cytosolic isotypes of the mutated yeast tRNA^{His} were compared with the respective profiles of trypanosomal tRNA^{Lys} whose mitochondrial isotype is modified at the penultimate cytidine before the anticodon as shown before (11). A gap in the alkaline hydrolysis ladder is not only observed, as expected, in the mitochondrial sample of the endogenous tRNA^{Lys} (Fig. 3B) but also in the alkaline hydrolysis ladder of the mitochondrial fraction of yeast tRNA^{His}. In this case, however, the gap corresponds to two nucleotide modifications (see also Fig. 3C). In addition, a downward shift below the gap of the mitochondrial alkaline hydrolysis profile when compared to its cytosolic counterpart is observed. A possible explanation for that shift would be the presence of a bulky or charged cytosol-specific base or nucleotide modification in the extreme 3′-region of yeast tRNA^{His}.

In order to confirm the identity of the tRNA and to define which region of the molecule is affected by the mitochondria-specific nucleotide modifications, the yeast tRNA^{His} was subjected to enzymatic sequence analysis using the base-specific ribonucleases T1, U2, PhyM and an extracellular ribonuclease of *B.cereus* (Fig. 3C). As expected for an enzymatically determined RNA sequence there are some ambiguities, however, the positions of guanosines can be determined exactly. Therefore the obtained sequence can easily be verified to correspond to yeast tRNA^{His} (17). To determine the exact positions of the modifications, however, is difficult. It is clear that the uridine (position 33) before the anticodon is modified. The cytidine at position 32 is most likely modified as well but the compression of the alkaline hydrolysis profile in that region makes the interpretation less clear. Nevertheless the same two nucleotides of the yeast tRNA^{His} are affected by mitochondria-specific nucleotide modifications as are modified in endogenous trypanosomal tRNAs (11). The situation is similar to the trypanosomal tRNA^{Tyr} which contains two adjacent mitochondria-specific modifications concerning the ultimate and penultimate positions before the anticodon. In both cases, the yeast tRNA^{His} and the trypanosomal tRNA^{Tyr} the modification of the uridine at position 33 appears to be a bulky group which leads to compression of the alkaline hydrolysis ladder above the gap.

DISCUSSION

In *T.brucei*, except for very few compartment-specific tRNAs, the same set of tRNAs is found in both cytosolic and mitochondrial fractions as evidenced by two dimensional polyacrylamide gel electrophoresis (7). Cytosolic and mitochondrial isotypes of trypanosomal tRNA^{Lys}, tRNA^{Leu} and tRNA^{Tyr} have been analyzed in

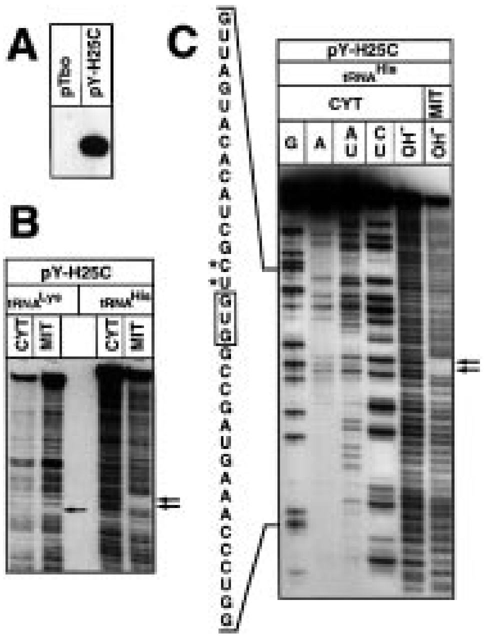

Figure 3. Yeast cytosolic tRNA^{His} becomes modified when imported into trypanosomal mitochondria. (**A**) Total RNA of cells transformed with pTbo not containing an insert was compared with cells transformed with pY-H25C expressing mutated yeast tRNA^{His}. The mutated tRNA^{His} was visualized by oligonucleotide directed 3′-splint labeling (see Materials and Methods). (**B**) Alkaline hydrolysis profiles of cytosolic (CYT) and mitochondrial (MIT) isotypes of 3′-splint labeled endogenous tRNA^{Lys} and yeast tRNA^{His}. Gaps indicative of nucleotide modifications are marked by arrows. (**C**) Gel-purified splint labeled yeast tRNA^{His} of cytosolic (CYT) and mitoplasts (MIT) fractions from the cell line transformed with pY-H25C were subjected to alkaline hydrolysis (OH^-) and RNA sequencing using base-specific RNases (cytosolic fraction) or to alkaline hydrolysis only (mitoplast fraction). The sequence indicated on the left corresponds to the predicted sequence for mutated yeast tRNA^{His}. The boxed nucleotides indicate the anticodon; the asterisk indicates the position of the nucleotide modification which is inferred from gap in the alkaline hydrolysis profile (arrow). G, RNase T1; A, RNase U2; A U, RNase PhyM; C U, RNase *B.cereus*.

more detail. The isotypes were shown to share the same sequence and to originate from the same gene (11,19). It is therefore difficult to assess the localization of trypanosomal tRNAs by cell fractionation only since most tRNAs are expected to be found in both the cytosolic and the mitochondrial fraction. Quantitative determination of the cytosolic cross-contamination for each mitochondrial preparation as well as the demonstration of nuclease resistance of the putatively imported tRNA within that fraction are essential. Unambiguous determination of the localization of a tRNA is indispensable for the analysis of the tRNA import pathway both *in vivo* or *in vitro*. Investigations of membrane translocation of macromolecules has in many other systems greatly been facilitated by the fact that the transported substrates are subject to covalent modifications during or shortly after transport. Targeting sequences are proteolytically removed from proteins destined to cross the mitochondrial, the chloroplast, the ER or the bacterial membranes (1). Also, small nuclear U RNAs after export from the nucleus acquire a hypermethylated cap structure which is involved in targeting of the RNAs for reimport (20).

I show here that the detection of mitochondria-specific nucleotide modification(s) by comparisons of alkaline hydrolysis profiles of specifically labeled cytosolic and mitochondrial isotypes of trypanosomal tRNAs offers an excellent tool to measure import of both homologous and heterologous tRNAs. As a model substrate I used a mutated version of the yeast cytosolic tRNA^{His} where the uridine at position 32 had been replaced by a cytidine. This tRNA was subject to mitochondria-specific nucleotide modifications affecting the same region as in the endogenous tRNA^{Tyr}. Indeed, limited sequence homology is found within and 3′ of the anticodon of trypanosomal tRNA^{Tyr} and the yeast tRNA^{His} (Fig. 1A) suggesting that some recognition elements of the modification enzyme responsible for the modification at position 33 may be located 3′ of the modified uridine.

The fact that the modified tRNAs are only found in mitochondria and that the nucleotide modifications are not involved in import, suggests that the modification enzymes are also localized within mitochondria. However, it cannot be formally excluded that the tRNAs are modified in the cytosol and immediately imported into mitochondria. Nevertheless, detection of a physical difference within a population of a tRNA species correlating with the cytosolic and the mitochondrial fractions is a powerful tool, analogous to proteolytic processing of proteins in other systems, to facilitate the analysis of both *in vivo* and *in vitro* tRNA import.

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