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In vivo import of unspliced tRNA^{Tyr} containing synthetic introns of variable length into mitochondria of *Leishmania tarentolae*

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ABSTRACT

The mitochondrial genomes of trypanosomatids lack tRNA genes. Instead, mitochondrial tRNAs are encoded and synthesized in the nucleus and are then imported into mitochondria. This also applies for tRNA^{Tyr}, which in trypanosomatids contains an 11 nt intron. Previous work has defined an exon mutation which leads to accumulation of unspliced precursor tRNA^{Tyr}. In this study we have used the splicing-deficient tRNA^{Tyr} as a vehicle to introduce foreign sequences into the mitochondrion of *Leishmania tarentolae*. The naturally occurring intron was replaced by synthetic sequences of increasing length and the resulting tRNA^{Tyr} precursors were expressed in transgenic cell lines. Whereas stable expression of precursor tRNAs^{Tyr} was obtained for introns up to a length of 76 nt, only precursors having introns up to 38 nt were imported into mitochondria. These results demonstrate that splicing-deficient tRNA^{Tyr} can be used to introduce short synthetic sequences into mitochondria *in vivo*. In addition, our results show that one factor which limits the efficiency of import is the length of the molecule.

INTRODUCTION

Import of tRNAs into mitochondria has been found in a wide variety of eukaryotes and has been studied in detail in *Saccharomyces cerevisiae*, in plants and in the protozoa *Tetrahymena*, *Leishmania* and *Trypanosoma brucei* (1). Using an *in vitro* import system and a collection of mutants, it has been shown that in yeast the single imported tRNA, tRNA^{Lys}(CUU), is transported via the protein import pathway using the mitochondrial precursor of lysyl-tRNA synthetase as a carrier (2,3). Not much is known about the mechanism of tRNA import in the other systems. In plants, a single nucleotide substitution in the acceptor stem of the nuclear encoded mitochondrial tRNA^{Ala} was shown to concomitantly abolish aminoacylation as well as import, suggesting that alanyl-tRNA synthetase is involved in the process (4). In *Leishmania* the situation looks quite different. Import of tRNAs into isolated mitochondria was shown to be dependent on ATP, but not on any cytosolic factors (5). A 15 kDa protein, which is partly localized in the mitochondrial fraction, has been implicated in the import process (6).

The number of mitochondrial tRNAs which are imported in the different species is quite variable. It ranges from one only in yeast, to a subset in plants, to the whole set in *Leishmania* and *T.brucei* (1). However, even in the latter cases cytosol-specific tRNAs were shown to exist (7–9), indicating that mitochondrial tRNAs have to be specifically targeted to the organelle. Analysis of the targeting signals in the different systems is complicated by the fact that the large majority of nuclear encoded mitochondrial tRNAs are found in the cytosol and the mitochondria (7,8). These tRNAs therefore perform a dual function in both cytosolic as well as mitochondrial translation. The best characterized import signal so far is the anticodon of imported tRNA^{Gln}(UUG) in *Tetrahymena*, which was shown to be necessary and sufficient for import (10). The importance of the anticodon as a targeting determinant has also been demonstrated in the imported tRNA^{Lys}(CUU) of yeast (1). In *Leishmania tarentolae*, however, the targeting signals appear to be localized in the D-loop of the tRNA, as evidenced by elegant *in vivo* experiments. It was shown that the cytosol-specific tRNA-^{Gln}(CUG) could be converted to an imported tRNA by introducing five point mutations into the D-loop of the tRNA (11).

In this study we investigated whether the intron-containing mutant of tRNA^{Tyr}(CUA) can be used as a vehicle to introduce foreign sequences into mitochondria of *L.tarentolae*.

MATERIALS AND METHODS

Cells and transfection

Wild-type and transformed *L.tarentolae* (UC strain) were grown at 27°C in Difco brain heart infusion medium containing 10 mg/ml hemin to late log phase (0.5×10^8–1.0×10^8 cells/ml).

Cells were transfected by electroporation using 10 µg of uncut plasmid each as described (12,13). After electroporation, cells

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were cultured for 24 h without selection before the addition of hygromycin B (Calbiochem, CA) to 75 mg/l.

Recombinant plasmids

All plasmids used for episomal expression are derivatives of pT-Y25 containing the splicing-deficient tRNA^{Tyr}(CUA) of *T.brucei* including 25 nt of 5′-flanking and 16 nt of 3′-flanking sequence followed by a PARP/procyclin promoter hygromycin resistance cassette (14). The intron of plamid pL-Y15 is flanked by *Cla*I and *Sal*I restriction sites, which served as cloning sites for insertion of the introns in constructs pL-Y33a, 33b, 55, 66, 76 and 230 (Fig. 1B). In the constructs pL-Y21, 38 and 59 the whole intron-containing tRNA^{Tyr} gene was synthesized *in vitro*.

Cell fractionation and RNA isolation

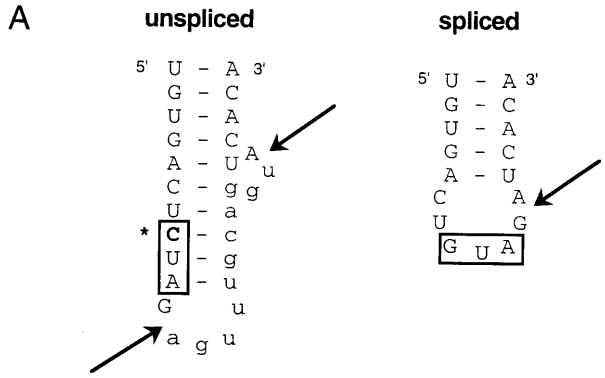
Mitoplasts were isolated after hypotonic lysis followed by ultracentrifugation in Urografin (Schering, Germany) gradients as described (15). RNA of total cells and cytoplasmic and mitochondrial fractions were purified by the acid guanidinium isothiocyanate method (16). Isolated RNA fractions were routinely treated with RNase-free DNase I (Boehringer Mannheim, Germany).

Northern blot analysis

The indicated amounts of cytosolic and mitochondrial RNA were separated on 8 M urea–8% polyacrylamide gels. Electrophoretic transfer and northern analysis were performed as described previously (14). Oligonucleotides were 5′-labelled using polynucleotide kinase and hybridizations were performed at high stringency, corresponding to a 55°C hybridization temperature. For the detection of unspliced and wild-type tRNA^{Tyr} the oligonucleotide 5′-TCCTTCCGGCCGGAATCGAAC-3′, hybridizing to the 3′-sequence of tRNA^{Tyr}, was used. For the cell line transformed with pL-Y59 the hybridization was performed with an oligonucleotide complementary to part of the intron sequence (5′-AGGTTCTCTACAACACCACAAC-3′). Cytosolic contamination was assessed using the oligonucleotide 5′-ACGCTCTCCGGGCGGAGAAA-3′, hybridizing to nt 92–111 of 7SL RNA.

RESULTS

The tRNAs^{Tyr} of *T.brucei* as well of *L.tarentolae* contain an 11 nt intron (9,17). Previous work in *T.brucei* using an *in vivo* import system has shown that an amber suppressing mutation within the anticodon of tRNA^{Tyr} leads to accumulation of unspliced precursor tRNA^{Tyr} (Fig. 1A; 18). Cell fractionation studies showed that this precursor tRNA^{Tyr} as well as the spliced wild-type tRNA^{Tyr} is imported into mitochondria (19). The fact that introns of up to 106 nt in length (20) were found in the tRNA^{Tyr} of some species suggests that the splicing-deficient tRNA^{Tyr} could be used as a vehicle to introduce foreign sequences into mitochondria of *L.tarentolae*. In order to test this approach, the naturally occurring intron of 11 nt was replaced in a first series of experiments by synthetic sequences of increasing lengths (15, 21, 38, 55, 66, 76 and 230 nt; Fig. 1B). The resulting tRNA^{Tyr} genes were then cloned into pTbo (21), a vector allowing episomal expression of tRNAs in trypanosomatids (14). Stable expression of splicing-deficient tRNAs^{Tyr} containing introns up to a length of 76 nt was obtained. However, no expression was



B

Plasmid	lengh of intron (nt)	Sequence of intron
pL-Y15	15	TCGATCGTAGTCGAC
pL-Y21	21	CAGAAACAAAGTTGTGGTGTT
pL-Y38	38	GTAGAGAACCTGGTCAAAAGACTAGGTATATA ATCTAT
pL-Y55	55	TCGATAAGAGGAGAGAAAAGAAAAGGCTTTAA CTTCAGGTTGTTTATGAGTCGAC
pL-Y66	66	TCGATAATAATTATAAAAGCGGAGAGAAAAGA AAAGGCTTTAACTTCAGGTTGTTTATGAGTCG AC
pL-Y76	76	TCGATTTAAATTTTAAATAATTATAAAAGCGG AGAGAAAAGAAAAGGCTTTAACTTCAGGTTGT TTATGAGTCGAC
pL-Y230	230	TCGATTTAAATTTTAAATAATTATAAAAGCGG AGAGAAAAGAAAAGGCTTTAACTTCAGGTTGT TTATTACGAGTATATGGTGTAGGTTTTAGTTT AGGTTTTTTTATTTGTATGCAAATTATATGTG GTGTTTGCTTAGCATGATTATTTTTTAGTTGC TTTATTTGTACAAATTGATATTTTGTATTATT TTTATGAGATTTTGATTTAGGATTTGTGATAC GTCGAC

Figure 1. (**A**) Predicted secondary structure of the anticodon stem–loop of unspliced tRNA^{Tyr} of *L.tarentolae*, containing the naturally occuring 11 nt intron (shown in lower case), and that of the spliced wild-type form. The amber suppressing mutation (G→C) leading to accumulation of unspliced tRNA^{Tyr} (18) is marked by an asterisk. Intron–exon boundaries are indicated by arrows. (**B**) Sequences of the synthetic introns inserted into the anticodon loop of the tRNA^{Tyr}. All constructs are based on pT-Y25 (14) containing a splicing-deficient version of tRNA^{Tyr}. Designation of plasmids is as follows: L, *Leishmania*; Y, tRNA^{Tyr}; the number indicates the length of the intron.

obtained from the construct having an intron of 230 nt, even though the presence of the insert-containing plasmid in the cell was demonstrated (not shown).

To test whether the unspliced tRNAs^{Tyr} expressed in the different cell lines are imported into mitochondria, RNA extracted from cytosolic and mitochondrial fractions of the transformed cell lines was prepared and analysed by northern hybridization (Fig. 2). The cell fractionation procedure used in

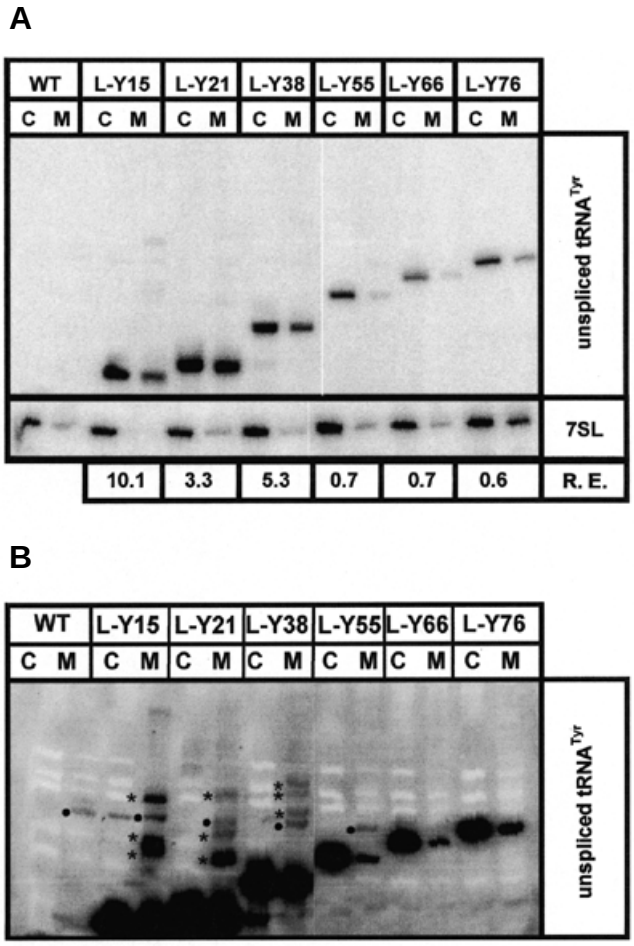


Figure 2. Import of unspliced $tRNA^{Tyr}$ variants into mitochondria of transfected cell lines. (**A**) Northern blot containing 4 µg of cytoplasmic (C) and 4 µg of mitochondrial (M) RNA was hybridized with a radioactively labelled oligonucleotide complementary to the 3′-end of $tRNA^{Tyr}$ as a probe. This oligonucleotide also detects the endogenous spliced $tRNA^{Tyr}$. Cell lines are named according to the plasmids used for transfection. WT, wild-type cells. The blot was subsequently reprobed with an oligonucleotide detecting the cytosol-specific 7SL RNA (lower). The relative enrichment (R.E.) of the $tRNA^{Tyr}$ was determined by comparing the ratios of the $tRNA^{Tyr}$ and the 7SL RNA in both the cytosolic and the mitochondrial fractions (details in Results). (**B**) Longer exposure of the same blot as in (A). Mitochondria-specific high molecular weight forms are indicated by asterisks, whereas the high molecular weight forms of the spliced wild-type $tRNA^{Tyr}$ are marked with dots.

these experiments is able to accurately detect whether a tRNA is imported or not. This has been shown in previous work, where the intracellular localizations of tRNAs were assessed using methods which are independent of the quality of the cell fractionation (14,22). It is, however, important to determine the cytosolic cross-contamination for each cell line individually due to the variation in the quality of the mitochondrial preparations. 7SL RNA, the RNA component of the signal recognition particle, has previously been shown to be a valid cytosolic marker in a comparable study in *T.brucei* (14). The contamination of the mitochondrial fraction with cytoplasmic material was quantified in each cell line by comparing the ratios of the tRNA and 7SL RNA signals in both the cytosolic and the mitochondrial fractions. The interpretation of the enrichment factor obtained by these calculations is as follows: any value >1 means that more of the tRNA is found in the mitochondrial fraction than expected for the chosen cytosolic marker. It is, however, known that 7SL RNA is partly associated with the endoplasmic reticulum, a known contaminant of mitochondrial preparations. It can therefore be expected that, when using 7SL RNA as a cytosolic marker, the cytosolic contamination is overestimated. In order to determine the value of the enrichment factor for a purely cytosol localized tRNA, a northern analysis of cytosolic and mitochondrial fractions of a cell line expressing a tagged variant of the only known cytosol-specific tRNA in *L.tarentolae*, $tRNA^{Gln}$(CUG) (9), was performed. In this experiment an enrichment factor of 0.6 was obtained (data not shown). Practically, this shows that a value of 0.6 means cytosolic localization of the tRNA, whereas values >0.6 indicate that it is imported. The wild-type cytosol-specific $tRNA^{Gln}$(CUG) would be the best cytosolic marker; however, it is not possible to directly use this tRNA as a marker, since in our hands all hybridization probes directed against it cross-hybridize with unknown imported tRNAs. Figure 2A shows that the precursor $tRNAs^{Tyr}$ containing introns of 15, 21 and 38 nt in length are clearly imported into mitochondria, giving enrichment factors of 3.3–10. The $tRNA^{Tyr}$ precursors with longer introns of 55, 66 and 76 nt, however, were not imported, as indicated by enrichment factors of 0.6–0.8. As expected, these factors agree well with those determined for the cytosolic $tRNA^{Gln}$(CUG). In Figure 2B, showing a longer exposure of the same northern blot as in Figure 2A, high molecular weight forms of the unspliced $tRNA^{Tyr}$ (indicated by asterisks) are seen. The nature of these high molecular weight forms is unclear. One possibility is that they represent circular derivatives due to the high RNA ligase activity found in trypanosomatid mitochondria (23). Whereas the exact structure of these forms is unknown, they have proven to be diagnostic for mitochondrially localized tRNAs and have never been detected in the cytosol (14,24). In agreement with that, we only observe these forms for the unspliced $tRNAs^{Tyr}$ (containing introns of 15, 24 and 38 nt), which are imported into mitochondria, but not for cytosolic contaminants. The band marked by a dot is also detected in mitochondria of wild-type cells and therefore does not originate from expression of transfected $tRNA^{Tyr}$ genes. In summary, these results suggest that there might be an upper length limit for import. However, since different nucleotide sequences were used as synthetic introns, it cannot be excluded that, besides the length, the nature of the sequence influences import. Two more experiments were performed in order to address this point. In the first one, two cell lines were prepared, expressing $tRNA^{Tyr}$ precursors containing 33 nt introns each. The two sequences essentially represent the two halves of the intron of plasmid pL-Y55 (Fig. 3A). Figure 3B shows that both of these precursor tRNAs were, unlike the one encoded on pL-Y55 (Fig. 2), imported into mitochondria. Their enrichment factors are 1.0 and 1.5 and therefore above the limit for a sole mitochondrial localization, which is 0.6. As with the previous cell lines, the mitochondrial localization of the $tRNA^{Tyr}$ precursors is confirmed by the detection of high molecular weight forms (Fig. 3B, asterisks). However, even though both $tRNA^{Tyr}$ precursors containing the shorter introns were imported, much less of the unspliced $tRNAs^{Tyr}$ were found in mitochondria than for the precursor tRNA encoded on pL-Y38, carrying an intron of comparable length (38 nt). Therefore, whereas the shorter length of the intron clearly stimulated import, the sequence of the

Figure 3. Import of tRNAs^{Tyr} containing fragments of the intron of the tRNA encoded in pL-Y55. (**A**) Synthetic introns inserted into the anticodon loop of tRNAs^{Tyr} in the cell lines L-Y33a and L-Y33b. The sequences are derived from the intron of the tRNA^{Tyr} encoded in pL-Y55. Designation of plasmids is as in Figure 1. (**B**) Northern blot containing 1.5 µg of cytoplasmic (C) and 4 µg of mitochondrial (M) RNA was hybridized with the same oligonucleotide as in Figure 2. The blot was subsequently reprobed with an oligonucleotide detecting the cytosol-specific 7SL RNA (lower). The relative enrichment (R.E.) of tRNA^{Tyr} was determined as in Figure 2. The arrow indicates the unspliced tRNAs^{Tyr}. Mitochondria-specific high molecular weight forms are indicated by asterisks, whereas the high molecular weight forms of the spliced wild-type tRNA^{Tyr} are marked with dots.

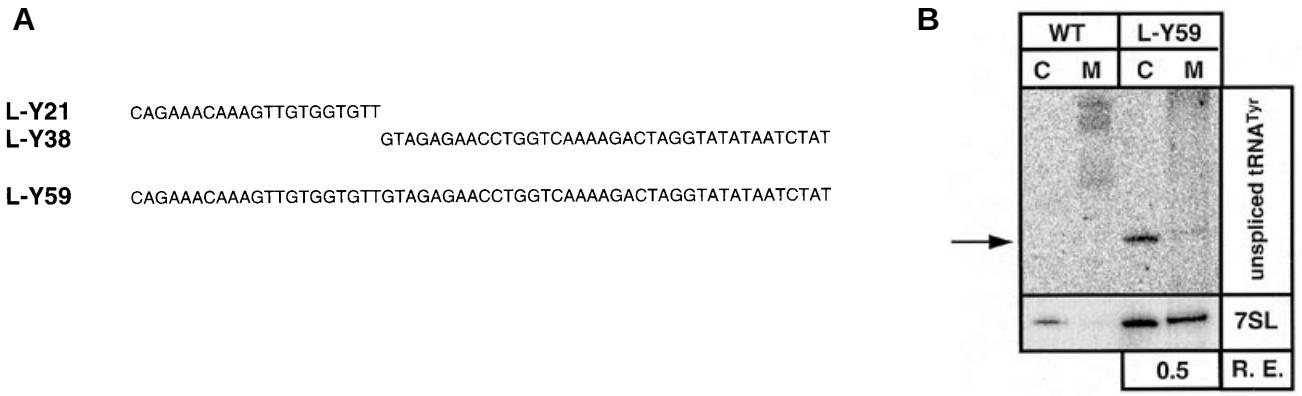


Figure 4. Import of tRNA^{Tyr} containing the combined introns of the tRNA encoded by pL-Y21 und pL-Y38. (**A**) Synthetic intron inserted into the anticodon loop of tRNAs^{Tyr} in the cell line L-Y59. The sequence is derived from the introns of tRNA^{Tyr} encoded in pL-Y21 and pL-Y38. Designation of plasmids is as in Figure 1. (**B**) Northern blot containing 1.5 µg of cytoplasmic (C) and 4 µg of mitochondrial (M) RNA was hybridized with an oligonucleotide hybridzing to the intron. The blot was subsequently reprobed with an oligonucleotide detecting the cytosol-specific 7SL RNA (lower). The relative enrichment (R.E.) of the tRNA^{Tyr} was determined as in Figure 2. The arrow indicates the unspliced tRNAs^{Tyr}. No high molecular weight forms are detected.

pL-Y55 intron does not appear to be as compatible with import as the one in pL-Y38.

These observations were confirmed in a second experiment where the converse approach was taken. The two introns of the precursor tRNAs^{Tyr} expressed from the pL-Y21 and pL-Y38 plasmids were combined and inserted into the new construct pL-Y59 (Fig. 4A). The expressed tRNA containing the 59 nt intron could not be imported into mitochondria, as judged from the enrichment factor of 0.5 (Fig. 4B). In agreement with that, no high molecular weight forms were observed.

In summary, these results support the conclusion that length of the intron is an important determinant influencing the import efficiency of the tRNA^{Tyr} precursor. In addition, however, it also appears that the intron sequence itself is affecting import.

DISCUSSION

Mitochondria of trypanosomatids have been the focus of much research. RNA editing especially has been studied extensively. However, due to the lack of a mitochondrial transfection system, most of the work has been confined to descriptive and *in vitro* studies (25–27). Mitochondria of trypanosomatids are known to import all of their mitochondrial tRNAs. Therefore, tRNA import might be used to study mitochondrial RNA editing by importing synthetically designed sequences expected to undergo RNA editing. tRNAs are generally synthesized as precursors having both 5′- and 3′-extensions (28). After transcription these extensions are enzymatically cleaved. Thus foreign sequences destined to be imported in the context of a tRNA cannot be fused to the 5′- or

the 3′-end but have to be inserted internally. We show that this is indeed possible by replacing the naturally occurring intron in $tRNA^{Tyr}$ with the desired synthetic sequences. The approach was facilitated by the fact that a splicing-deficient $tRNA^{Tyr}$ carrying a point mutation within the anticodon has been described (18). In addition, previous work has shown that the anticodon loop, where the introns are inserted, is not a crucial determinant for import (19). Here we show that unspliced $tRNA^{Tyr}$ containing introns up to a length of 38 nt can be imported into mitochondria. Some of the sequences used as synthetic introns were designed to represent substrates for RNA editing. However, in no case was any evidence for RNA editing within the introns obtained. One possible reason for this negative result could be that the introns have only been presented to the RNA editing machinery in the structural context of a tRNA. Whereas our attempt to study RNA editing by importing artificial RNA substrates failed, unspliced $tRNA^{Tyr}$ will be useful to import short synthetic RNA sequences for other purposes. Import of short synthetic sequences could potentially be used to import hammerhead ribozymes targeted to cleave specific mitochondrial RNAs (29). Interestingly, we found that the length of the intron influences import. Clearly, longer introns were not as compatible with import as shorter sequences. The upper length limit for the inserted intron appears to be ~40 nt. However, import also depends on the sequence of the intron. tRNAs encoded in pL-Y33a, pL-Y33b and pL-Y38 were imported with very different efficiencies, even though they contain introns of comparable length. The large influence of the intron length and sequence on import is an unexpected finding, since only the D-loop of tRNAs has been shown to contain targeting information in *Leishmania* so far (11). However, all the unspliced $tRNAs^{Tyr}$ used in our study contained identical wild-type D-loop structures. A possible interpretation of these results is that, in addition to the D-loop, some other structural feature of the tRNA is essential for import and that this structure is disturbed in $tRNAs^{Tyr}$ containing introns which are not imported. In agreement with this interpretation it has been shown that even though a cytosol-specific $tRNA^{Gln}$ having the D-loop of the efficiently imported $tRNA^{Ile}$ is imported into mitochondria, the converse experiment failed, $tRNA^{Ile}$ remaining mitochondrial even after swapping its D-loop with that of the cytosolic $tRNA^{Gln}$ (11). This shows that the targeting information is not confined to the D-loop only. In order to determine the relative contribution of length and structure of the imported substrates to mitochondrial targeting, we must await future studies defining the precise tRNA targeting signals in trypanosomatids.

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