The T-Stem Determines the Cytosolic or Mitochondrial Localization of Trypanosomal tRNAsMet

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The mitochondrion of Trypanosoma brucei lacks tRNA genes. Organellar translation therefore depends on import of cytosolic, nucleus-encoded tRNAs. Except for the cytosol-specific initiator tRNA Met, all trypanosomal tRNAs function in both the cytosol and the mitochondrion. The initiator tRNA Met is closely related to the imported elongator tRNA Met. Thus, the distinct localization of the two tRNAs Met must be specified by the 26 nucleotides, which differ between the two molecules. Using transgenic T. brucei cell lines and subsequent cell fractionation, we show that the T-stem is both required and sufficient to specify the localization of the tRNAs Met. Furthermore, it was shown that the tRNA Met T-stem localization determinants are also functional in the context of two other tRNAs. In vivo analysis of the modified nucleotides found in the initiator tRNA Met indicates that the T-stem localization determinants do not require modified nucleotides. In contrast, import of native tRNAs Met into isolated mitochondria suggests that nucleotide modifications might be involved in regulating the extent of import of elongator tRNA Met.

INTRODUCTION

In most protozoa, many fungi, in plants, and even in a few animals, the number of mitochondria-encoded tRNA genes is not sufficient to support mitochondrial translation. It has been shown in these organisms that the missing mitochondrial tRNA genes are compensated for by import of a small fraction of the corresponding cytosolic tRNAs (Schneider and Maréchal-Drouard, 2000). The number of tRNAs that are imported depends on the species. The most extreme cases are Saccharomyces cerevisiae whose mitochondria import a single tRNA only (Tarassov and Martin, 1996) and two groups of parasitic protozoa, the trypansomatids (such as Trypanosoma brucei and Leishmania) (Simpson et al., 1989; Hancock and Hajduk, 1990) and the apicomplexans (such as Plasmodium falciparum) (Feagin, 2000), both of which completely lack mitochondrial tRNA genes and therefore must import the whole set of tRNAs from the cytosol. Plants are in between; they import a significant fraction of their mitochondrial tRNAs, but they still have mitochondria-encoded ones (Dietrich et al., 1992). Interestingly, an imported nucleus-encoded mitochondrial tRNA always only represents a small fraction (typically 5%) of a normal cytosolic tRNA. Two basic questions in regard to mitochondrial tRNA import therefore are 1) What determines which tRNAs are imported and which ones remain in the cytosol, and 2) Why is it always only a small fraction of a given tRNA that is imported into mitochondria?

The first question concerning the specificity of tRNA import has been most extensively studied in S. cerevisiae and Tetrahymena thermophila. In yeast, one of two cytosolic tRNA Lys isoacceptors is imported into mitochondria (Martin et al., 1979). Import occurs in complex with the precursor of mitochondrial lysyl-tRNA synthetase via the protein import pathway (Tarassov et al., 1995). Specificity of import is achieved by binding to the lysyl-tRNA synthetase, it requires amino-acylation and specific sequence elements in the acceptor stem and the anticodon loop of the imported tRNA (Rusconi and Cech, 1996). Specificity of import in plants and trypanosomatids: a point mutation in the acceptor stem of tRNAAla of potato was shown to abolish import (Dietrich et al., 1996). In Tetrahymena, one of three nucleus-encoded homologous tRNACys isoacceptors, the tRNACys UUG, is imported into mitochondria (Rusconi and Cech, 1996a). Only fragmentary results are available for what determines the specificity of tRNA import in plants and trypanosomatids: a point mutation in the acceptor stem of tRNAVal of potato was shown to abolish import in vivo (Dietrich et al., 1996) and more recently the D-loop and the anticodon region were implicated in import of plant tRNAVal (Delage et al., 2003). In Leishmania, some studies suggest that the D-stem loop (D-arm) region of tRNAs might contribute to the localization determinants (Lima and Simpson, 1996; Mahapatra et al., 1998). Furthermore, analyses of randomized RNA substrates selected for import competence in an in vitro system have been performed but failed to recover sequence elements that could be clearly attributed to specific regions of existing tRNAs (Bhattacharyya et al., 2002).

The second question of how the extent of import is regulated has only recently been addressed. It was shown that in T. brucei the fraction of a given tRNA that is found in the mitochondrion ranges from 1 to 8% and is independent of the expression level of the tRNA (Tan et al., 2002b). Comparative analysis of cytosolic and mitochondrial fractions of various trypanosomal and leishmanial tRNAs revealed

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physical differences in the two populations: mitochondria-specific (Schneider et al., 1994b; Crain et al., 2002) as well as of cytosol-specific nucleotide modifications (Kaneko et al., 2003) were detected. Further experiments suggested that the mitochondria-specific modifications in the anticodon loop are not responsible for mitochondrial targeting but rather reflect a consequence of import (Schneider et al., 1994b). Interestingly, this seems to be different for the cytosol-specific 2-thiouridines, which are found in the anticodon wobble position of leishmanial tRNA^Gln(UUC) and tRNA^Gin (UUG), because the two tRNAs carrying these modifications were prevented from being imported in an in vitro system (Kaneko et al., 2003).

All mitochondrial tRNAs in T. brucei are imported from the cytosol. However, a single cytosol-specific tRNA has been identified. This tRNA corresponds to the eukaryotic initiator tRNA^Met (tRNA^Met-1) and its cytosolic localization might be due to the fact that it cannot function in the context of the bacterial-type translation system of the mitochondrion (Tan et al., 2002a). The tRNA^Met-1 is highly homologous to the elongator tRNA^Met (tRNA^Met-e), which as all other trypanosomal tRNAs is in part (5.5% of the cellular content) imported into mitochondria (Tan et al., 2002b). In the present study, we have determined which nucleotides are responsible for the differential in vivo localization of the two tRNAs^Met. In addition, it was shown that the same nucleotides also function in the context of two other tRNAs. Furthermore, we present evidence that contrary to the specific cytosolic localization of the tRNA^Met-1 the extent of import of tRNA^Met-e might be mediated by nucleotide modifications.

**MATERIALS AND METHODS**

**Cells**

Protoplasts from T. brucei, stock 427, grown at 27°C in SDM-79 medium supplemented with 5% fetal bovine serum. Cells were harvested at late log phase (corresponding to 2.5 × 10^7 to 3.5 × 10^8 cells/ml and washed once in cold 20 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl and 20 mM glucose. The resulting cellular pellets were either used to isolate total RNA or prepared for mitochondrial isolation from the pellets. RNA from total cells or isolated mitochondrial fractions of wild-type and transgenic cell lines were prepared that allow stable integration into the trypanosomal rDNA loci (Biebinger et al., 2003). The resulting cellular pellets were either used to isolate total RNA or prepared for mitochondrial isolation by digitonin extraction.

**Transfection of T. brucei**

For expression of the tRNA variants derivatives of the pHD437 plasmid were used that harbor the lacZ-α integrase into the trypanosomal tRNA loci (Biebinger et al., 1996). The Km/ß-gal fragment of the plasmid was replaced by inserts containing the variant tRNA genes. Variations in tRNA genes were introduced by PCR-mediated mutagenesis and verified by sequencing. All variant tRNA fragments were expressed containing 261 nucleotides of the 5′-flanking region of the tRNA^Met (CAG) gene and their own 3′-flanking region. The constructs were linearized with NotI, electroporated into T. brucei and transformants were selected with phleomycin and cloned as described previously (Beverley and Clayton, 1993).

**Analysis of In Vitro Import**

Mitochondrial fractions of wild-type and transgenic cell lines were prepared by digitonin extractions (Tan et al., 2002b). Washed cells (2 × 10^8 cells each) were resuspended in 0.5 ml of SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA). Five percent of the sample (25 µl) was removed to isolate the total RNA. After the addition of 0.475 ml of SoTE containing 0.067% (wt/vol) digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The suspension was centrifuged (8000 g/5 min/4°C), and the supernatant was discarded. Next, the resulting pellets were resuspended in 500 µl of SoTE containing 1 µg of RNase A and incubated on ice for 15 min. After a final centrifugation, the supernatants were discarded, and RNA was isolated from the pellets. RNA from total cells or isolated mitochondrial fractions was purified by the acidic guanidinium thiocyanate method as described previously (Chomczynski and Sacchi, 1987). Isolated total (0.5 × 10^8 cell equivalents) and mitochondrial RNA (10^6 cell equivalents) were resolved on 8 M urea/10% polyacrylamide gels and processed for Northern analysis as described previously (Tan et al., 2002b). Transgenic tRNAs were detected by specific hybridization by using 5′-end-labeled oligonucleotides. The following oligonucleotides were used to detect the indicated tRNAs or tRNA variants: wild-type tRNA^Met-e, 5′GGGGCTCGAACCCA3′; tRNA^Met-e variants carrying the tagged D-arm of the tRNA^Met-e, 5′GGGGCTCGAACCCA3′; tRNA^Met-e variants carrying the tagged D-arm of the tRNA^Met-e, 5′CGGCTCTGCCGACTGAGCC3′; wild-type tRNA^Met-e, 5′GGGTGCCAACCCTGGGCGTCAACCA3′; variant tRNA^Met-e, 5′CAACGCTTCAAGGTGTA3′; wild-type tRNA^Met-e, 5′TGCTCCGGCGGGTGCAAA3′; and variant tRNA^Met-e, 5′CAACGCCTCTCGGTC3′.

**Isolation of Native tRNAs^Met-1**

To isolate native, fully modified tRNAs, tRNA^Met-e and tRNA^Met-e present in total RNA fraction (5 µg) and the tRNA^Met-e present in the mitochondrial RNA fraction (5 µg) were labeled by using the highly efficient and specific 3′-end-labeling protocol. In this method, an oligonucleotide (5′AGTGTTGCGATCCGTAGGCTCGAACCCA3′ for tRNA^Met-e and 5′CAACGCTTCAAGGTGTA3′ for tRNA^Met-e) was hybridized to the 3′-end of the SoTE-containing RNA where it leaves a 5′ overhang, the complementary nuclease (in our case, a dCTP) to the 3′ end of the selected tRNA (Schneider et al., 1994b). The labeled RNA fractions were then separated on an 8 M urea/10% polyacrylamide gel, the labeled band was localized, cut out, and eluted from the gel.

**Analysis of In Vitro Import**

A standard in vitro import reaction was performed in 16 µl of SoTE containing 2 mM dithiothreitol, 20 mM MgCl_2 and isotopically isolated mitochondrion (200 µg of protein) (Hasuer et al., 1996). After the addition of 2 µl of substrate RNA the reaction was incubated for 10 min at 27°C in either the absence or the presence of a mixture containing 2 mM ATP, 1 µM amylose, 0.5 M creatine phosphate, and 0.7 µCi of [γ-32P]ATP (DuPont, Indianapolis, IN). Subsequently, CaCl_2 was added to a final concentration of 2 mM, and the reaction was digested with 45 U of micrococcal nuclease (MBI Fermentas, Lithuania) for 30 min at 37°C. Finally, 1 µl of SoTE containing 4 mM EGTA and 4 mM EDTA was added. The mitochondrial pellet was resuspended in 100 µl of 10 M Tris-Cl, pH 7.5, containing 1 mM EDTA and 0.2% SDS. RNA was isolated by phenol extraction and alcohol precipitation and analyzed 8 M urea/10% polyacrylamide gels.

**Separation of RNAs by two-dimensional (2D)-PAGE and dot blotting were performed according to Maniatis et al. (1982).**

**RESULTS**

Previous studies have shown that the mitochondrial localization of trypanosomal and leishmanial tRNAs does not depend on a specific 5′-genomic context (Hauser and Schneider, 1995; Kapushoch et al., 2000; Tan et al., 2002b). Therefore, because the cytosolic tRNA^Met-1 and the imported tRNA^Met-e differ by 26 nucleotides only (Figure 1A), these differences or a subset thereof must be responsible for their differential localization. To determine which of these sequence elements are critical for localization, transgenic T. brucei cell lines were prepared that express variants of tRNAs^Met-e carrying distinct domains of the imported tRNA^Met-e and vice versa. Introduction of a tag (switching the base pair G12/C23 to U12/A23; Figure 1A) allowed specific detection of the transgenic tRNA^Met-e by oligonucleotide hybridization. The presence of the tag does not alter the localization of the cytosol-specific tRNA^Met-e nor that of the imported tRNA^Met-e (unpublished data). All transgenic tRNAs were expressed in the 5′-genomic context corresponding to the 5′-flanking sequence of the tRNA^Met-e (CAG) because this sequence allowed efficient expression of all tRNA variants but does not influence their intracellular localization.
In Vivo Localization of tRNA_{Met-i} Variants

In a first series of experiments cell lines expressing variants of tRNA_{Met-i} carrying the acceptor stem, the D-arm, the anticodon stem loop or the T-stem loop (T-arm) of the imported tRNA_{Met-e} were produced. Total and mitochondrial RNA from the transgenic cell lines where analyzed for the presence of the corresponding tRNA_{Met-i} variants by using specific oligonucleotide hybridization. Figure 1B shows that the T-arm of the imported tRNA_{Met-e} is sufficient to confer a mitochondrial localization to the normally cytosol-specific tRNA_{Met-i}. The three other domains of the tRNA_{Met-e} do not seem to contain targeting information. The bottom two panels of Figure 1B show the intracellular distribution of wild-type tRNA_{Met-i}, which serves as cytosolic marker, and that of wild-type tRNA_{Met-e}, respectively. Comparison of the hybridization signals on the top and bottom panels of the right column of Figure 1B indicate that the tRNA_{Met-i} variant that carries the T-arm of the tRNA_{Met-e} is imported with comparable efficiency than the wild-type tRNA_{Met-e}. In a further analysis, we tested whether the whole T-arm is required for targeting of tRNAs_{Met}. Four cell lines expressing variants of tRNA_{Met-e} carrying the T-loop (1), the T-stem together with the variable loop (2), the T-stem only (3), or the single T-stem nucleotide pair A52:62U (4) of the imported tRNA_{Met-e} were established. Analysis of total and mitochondrial RNA of the four transgenic cell lines indicates that it is the T-stem alone that contains the localization determinants (Figure 1C).

The T-stem of the tRNA_{Met-e} and the tRNA_{Met-e} differ by two nucleotide pairs only (Figure 1A). Further dissection of the T-stem localization determinants shows that the tRNA_{Met-i} variant carrying the single tRNA_{Met-e}-derived nucleotide pair A52:62U remained in the cytosol (Figure 1C, far right), whereas the tRNA_{Met-i} variant carrying the tRNA_{Met-e} nucleotide pair G51:63C was imported, albeit less efficiently than the wild-type tRNA_{Met-e} (Figure 3B, right). Note that the tRNA_{Met-i} variant carrying the G51:63C nucleotide pair of the tRNA_{Met-e} is identical to the tRNA_{Met-i} variant carrying the T-stem of the tRNA_{Lys}. This suggests that it is the 51:63 nucleotide pair that plays the most important role in localization. The imported tRNA_{Met-i} variants carrying the T-stem of the tRNA_{Met-e} seem to be few nucleotides shorter than their cytosolic counterparts. The reason for this is not clear, but a possible explanation would be that the tRNA_{Met-i}, which in vivo is only found in the cytosol, becomes truncated when present in the mitochondrion. Alternatively, the mobility shift might be caused by some mitochondria-specific nucleotide modification. Interestingly, however, the same shift was not observed for the tRNA_{Met-i} variant shown in Figure 3B, right.

Finally, we also did the converse experiments. Variants of the tRNA_{Met-e} carrying the T-arm or the T-stem only of the cytosolic tRNA_{Met-i} were expressed and shown to be largely retained in the cytosol (Figure 2). It should be mentioned, though, that some residual import is still observed for the variant tRNA_{Met-e} that carries the T-stem of the tRNA_{Met-i} (Figure 2, right), indicating a minor but detectable role of the T-loop in the localization of the tRNAs_{Met}.

In summary, the in vivo results show that the T-arm of the trypanosomal tRNAs_{Met} is both necessary and sufficient to specify the localization of the tRNA_{Met}.

In Vivo Localization of Variant tRNA_{Lys} and tRNA_{Ile}

Do the T-arm localization determinants of the tRNA_{Met-i} also function in the context of other tRNAs? To test this, we chose the tRNA_{Lys} of which 3.5% and the tRNA_{Ile} of which 8.5% are imported into mitochondria (Tan et al., 2002b). Cell lines were established that express variants of the two tRNAs that carry the T-arm of the cytosolic tRNA_{Met-i} (Figure 3A). Cell fractionation and Northern analysis show that both of these molecules are retained in the cytosol (Figure 3B and C, left column). Furthermore, we also prepared cell lines expressing variant tRNAs_{Met-i} carrying the T-stem of either the tRNA_{Lys} (as discussed above, this tRNA is identical to the
tRNA\textsuperscript{Met-i} variant carrying the G51:C63 of the tRNA\textsuperscript{Met-e}) or the tRNA\textsuperscript{Leu} and showed that both of these were imported into mitochondria. However, whereas the T-stem of the tRNA\textsuperscript{Lys} induced import whose extent was comparable with that of wild-type tRNA\textsuperscript{Lys} (Figure 3B, right column), the tRNA\textsuperscript{Met-i} containing the T-stem of the tRNA\textsuperscript{Leu} was less efficiently imported than wild-type tRNA\textsuperscript{Leu} (Figure 3C, right column).

Localization of tRNA\textsuperscript{Met-i} Is Independent of Nucleotide Modifications

It was recently shown that cytosol-specific 2-thiouridines that are found in the anticodon wobble position of leishmanial tRNA\textsuperscript{Glu}UUU and tRNA\textsuperscript{Gln}UUG prevent in vitro import of these tRNAs (Kaneko et al., 2003), suggesting that nucleotide modifications may act as anti-import determinants. To test whether modified nucleotides might also be responsible for the exclusive cytosolic localization of the tRNA\textsuperscript{Met-i}, we determined the nucleotide modification in the T-arm region of T. brucei wild-type tRNA\textsuperscript{Met-i}. To that end, the tRNA\textsuperscript{Met-i} was isolated by 2D-gel electrophoresis (Figure 4A) and sequenced using the technique described by Stanley and Vassilenko (1978). This method allows to simultaneously identify the position and the nature of most modified nucleotides (for details, see MATERIALS AND METHODS). The results in Figure 4B show that nucleotide modifications were detected at position 55 (corresponding to a pseudouridine) and at position 58, which according to 2D TLC (Nishimura, 1979), most likely corresponds to a derivative of 1-methyladenosine [m(1)A] (our unpublished data). The nucleotide at position 46 in the variable loop could not be determined due to a compression of the nucleotide ladder in the first dimension. Most importantly, however, it is clear that no modified nucleotides are present within the T-stem itself. Furthermore, none of the detected modifications in the T-loop are expected to be specific for the tRNA\textsuperscript{Met-i}. Indeed, the pseudouridine probably occurs in all tRNAs (Sprintzel et al., 1996), and a m(1)A at position 58 has been detected in many different tRNAs, including the tRNA\textsuperscript{Met-i} and tRNA\textsuperscript{Leu} (Anderson et al., 2000). Thus, it is unlikely that changing the two T-stem nucleotide pairs that are responsible for the in vivo localization of tRNA\textsuperscript{Met-i} will have any effect on the modification pattern of the tRNA\textsuperscript{Met-i} variants. To confirm that the in vivo localization determinant does not correspond to a single nucleotide modification, we tested the in vivo import of a mutant tRNA\textsuperscript{Met-i} carrying an A-to-U replacement at position 58. The transgenic tRNA\textsuperscript{Met-i} lacks the m(1)A derivative at position 58 but nevertheless is correctly localized in vivo (Figure 4D, left). A survey of the literature shows that the only nucleotide modification described for the T-stem is found in tRNAs\textsuperscript{Met-i} of yeast and plants and consists of an unusual 2'-O-phosphoribosyl purine [Rp(p)] at position 64. The function of Rp(p) is to prevent binding of the tRNA\textsuperscript{Met-i} to eukaryotic elongation factor 1 (eEF1) (Forster et al., 1993; Astrom and Bystrom, 1994). Rp(p) is a large and complex modification that due to chemical instability might have been missed in our analysis shown in Figure 4B. To exclude this, we produced a cell line expressing a mutant tRNA\textsuperscript{Met-i} containing the inverted base pair 50A:64U instead of the wild-type 50U:64A. This tRNA\textsuperscript{Met-i} variant lacks any putative Rp(p) modification but nevertheless remains cytosol specific (Figure 4D, right).

In Vitro Import of Isolated tRNA\textsuperscript{Met}

Analysis of transgenic trypanosomes expressing different tRNA\textsuperscript{Met} variants allowed to identify the two adjacent un-
which were introduced in the respective tRNAMet-i gene of the position 58, and the 50U:64A to A50:U64 nucleotide pair switch, A-to-U replacement introduced to abolish the m(1)A derivative at tRNAMet-i showing the position of the modified T-stem nucleotide pairs (51:63 and 52:62) as the determinants for in vivo localization. In a next step, we tested whether tRNAMet-i variants that are in vivo either cytosolic or in part mitochondrially localized can be imported into isolated mitochondria. Preliminary experiments showed that in vitro transcripts of all tRNAsMet were imported (our unpublished data). Import of in vitro transcribed tRNAMet-i is unexpected, because in vivo the tRNA is cytosol specific. At present, we cannot explain these results. However, it is unlikely that in vitro import of tRNAMet-i is due to the lack of nucleotide modifications in the in vitro transcript because the in vivo localization determinants of tRNAMet-i do not consist of modified nucleotides (Figure 4). A potential trivial explanation for the results might therefore be that in vitro transcripts cannot efficiently adopt a native-like tRNA structure. In any case, these results show that, if in vitro-transcribed tRNAs are used for in vitro import experiments, the results may not automatically be applicable to the in vivo situation.

If isolated instead of in vitro transcribed tRNAsMet were used for in vitro import, the obtained results are compatible with the in vivo analysis. No import was observed for either the tRNAMet-e or the tRNAMet-i isolated from cytosolic RNA, whereas the tRNAMet-e isolated from the mitochondrial fraction was imported (Figure 5). Because the cytosolic and the mitochondrial tRNAMet-e derive from the same gene, these results suggest that, although nucleotide modifications are not involved in the localization of the tRNA Met-i (Figure 4), they may in analogy to the situation for tRNA Glu/Gln in Leishmania tarentolae (Kaneko et al., 2003) be responsible for the lack of import of isolated cytosolic tRNA Met-e.

**DISCUSSION**

**In Vivo Import Experiments**

Previous in vivo studies of mitochondrial tRNA targeting in trypanosomatids have demonstrated that the localization determinants are localized within the coding region of the tRNAs (Hauser and Schneider, 1995; Kapushoc et al., 2000; Tan et al., 2002b). In addition, evidence was presented that aminocyl-tRNA synthetases are not involved in import, because an intron-containing tRNA Tyr that cannot be aminocylated still was imported (Schneider et al., 1994a). Not much success was reported in identifying which regions within a tRNA might contain the localization information. That fact that intron-containing tRNA Tyr was imported showed that large insertions are tolerated in the anticodon loop. Furthermore, in vivo studies in L. tarentolae suggested...
that the D-arm may contain targeting information, because a variant of the cytosol-specific tRNA^{Cys}(CUG) containing the D-arm of the imported tRNA^{Leu} could be imported. However, the interpretation of these results is complicated by the fact that the converse experiment was not successful and that a tRNA^{Asp} variant containing the D-arm of the cytosolic tRNA^{Cys}(CUG) was still imported (Lima and Simpson, 1996).

Our analysis of in vivo import of trypanosomal tRNAs^{Met} confirms that the targeting determinants are localized within the coding sequence. Furthermore, the fact that tRNA^{Met+e} and tRNA^{Met+i}, despite their distinct intracellular localizations, are aminoacylated by the same enzyme suggests the methionyl-tRNA synthetase is not involved in import. Most importantly, our study identified the two T-stem nucleotide pairs (51:63 and 52:62) as elements that are both necessary and sufficient to specify the mitochondrial or cytosolic localization of the trypanosomal tRNAs^{Met}. Further dissection of the elements suggests that the 51:63 is the more important of the two nucleotide pairs. T-stem localization determinants of the tRNA^{Met+i} were also functional in the context of the tRNA^{Asp} and tRNA^{Leu}. The tRNA^{Leu} is the same tRNA whose mitochondrial localization in 

In Vitro Import Experiments

In summary, our work, for the first time, identified a specific region in a T. brucei tRNA that 1) when transplanted from a cytosolic to an imported tRNA, prevents its import; and 2) when transplanted from an imported to a cytosolic tRNA, induces its import.

How is the specificity of tRNA import determined in other organisms? The best studied case is Tetrahymena where a quantitative in vivo analysis has shown that the anticondon UUG of the imported tRNA^{Asp} is both necessary and sufficient to induce import of any of the three tRNAs^{Asp} isoc鄱ceptors (Rusconi and Cech, 1996a). However, whether the same determinant also works in the context of other tRNAs is not known. As discussed in Introduction, import of the tRNA^{Lys} into yeast mitochondria requires aminoacylation and specific sequence elements in the acceptor stem and the anticodon loop (Entelis et al., 1998). Recent work in higher plants indicated the importance of the D-arm as well as the anticondon for in vivo localization of the tRNA^{Val} (Delage et al., 2003). Thus, these results illustrate that, as might be expected due to the polyphyletic evolutionary origin of mitochondrial tRNA import (Schneider and Marechal-Drouard, 2000), the in vivo localization determinants are not conserved between different species.

Figure 6. Alignments of T-arm sequences of all known T. brucei tRNAs. tRNA^{Met+e} and tRNA^{Met+i} are listed at the top. tRNA^{Asp} and tRNA^{Leu}, which were used in the in vivo experiments shown in Figure 3, are indicated by the asterisks. Sequences were taken from http://zoosun00.unifr.ch/Trypanos/WWW%20tRNA/tRNAGenOrg.html. The tRNA^{Asp} is cytosolic; all other tRNAs are in part imported. Nucleotides in the T-arms of the different tRNas, which are identical to the cytosolic localization determinant identified in the tRNA^{Met+e}, are boxed. With the exception of the tRNA^{Asp} (indicated by the arrow) the two nucleotide pairs (U51:A63 and G52:C62) occur only in the tRNA^{Met+i}.

patra et al., 1998), and based on these and other results, a consensus purine-rich D-arm motif was suggested to be a general import determinant (Bhattacharyya et al., 2002). The motif is claimed to be absent from yeast tRNAs, which were not able to compete for in vitro import of leishmanial tRNAs. However, the in vivo significance of the putative D-arm motif was questioned by a sequence analysis that failed to find a conserved D-arm motif, which as expected for an import determinant would consistently be present in imported trypanosomatid tRNAs but be absent from non-imported and yeast tRNAs (Suyama et al., 1998).

Finally, the interpretation of results obtained in in vitro assays is complicated by the fact that small RNA transcripts are imported independently of their sequence as was reported by two groups (Nabholz et al., 1999; Rubio et al., 2000).
In summary, it is clear that when in vitro systems are used to study the specificity of tRNA import in trypanosomatids, the results may not automatically be applicable to the in vivo situation. We believe that one reason for this might be the fact that in all discussed studies in vitro transcribed tRNAs were used as substrates in the in vitro assays, because by using isolated tRNAs as substrates (Figure 5), we have obtained results that are compatible with the in vivo situation.

**Import or Retention Signal?**

There are two principally different ways of how the T-stem localization determinants could function: 1) they could act as a retention signal and prevent import of cytosolic tRNA, or 2) they could act as a positive import signal in all imported tRNAs. In the first case, specificity might be mediated by a factor binding to tRNA\textsuperscript{Met} only, whereas in the second case we expect to find a factor that specifically binds imported tRNAs. The results of the in vitro import experiments shown in Figure 5 argue against a soluble retention factor because a comparable import specificity than in vivo is obtained in the reconstituted system even though it is devoid of cytosolic factors. However, it is possible that some factors remain associated with the mitochondrial membranes during isolation; therefore, no definitive conclusions can be drawn.

Interestingly, in *T. brucei* all elongator tRNAs are in part imported, whereas the single tRNA\textsuperscript{Met} is not (Tan et al., 2002b). Could the import specificity therefore be linked to the initiation or elongation function of tRNAs? For a tRNA\textsuperscript{Met} to be functional in initiation it needs 1) to interact with eukaryotic initiation factor 2 (eIF2) (Farruggio et al., 1996), and 2) to be excluded from binding to eEF1 (Drabkin et al., 1998). Elongator tRNAs, on the other hand, selectively interact with eEF1. In trypanosomes, the situation is complicated by the fact that *T. brucei* elongator tRNAs, because they are used in cytosolic and mitochondrial translation, are expected to bind to both the cytosolic eEF1 and its mitochondrial homologue, elongation factor Tu (EF-Tu). Should the T-stem localization determinants in the tRNA\textsuperscript{Met} act as a retention signal, it could be the interaction of the tRNA\textsuperscript{Met} with the eIF2 that prevents import. We believe that this is not very likely because a tRNA\textsuperscript{Met} variant carrying the acceptor stem of the tRNA\textsuperscript{Met} remains in the cytosol (Figure 1B). The A1–U72 nucleotide pair of the acceptor stem, however, is a specific hallmark of eukaryotic tRNA\textsuperscript{Met} and one of the most important determinants for binding to eIF2 (Farruggio et al., 1996). If we assume, on the other hand, that the T-stem localization determinants in the elongator tRNAs act as a positive import signal, import specificity might be mediated by binding to cytosolic eEF1 or to the mitochondrial precursor of EF-Tu. The main determinants responsible for preventing the binding of vertebrate tRNA\textsuperscript{Met} to eEF1 are nucleotides (A50:U64 and U51:A63), which are thought to introduce a sequence-specific perturbation of the sugar phosphate backbone in the T-stem (Drabkin et al., 1998) and thus overlap with the main cytosolic T-stem localization determinant identified in our work, the nucleotide pair U51:A63. We therefore assume that also in *T. brucei* this nucleotide pair prevents interaction with either cytosolic eEF1 or the mitochondrial precursor of EF-Tu.

Surprisingly, the tRNA\textsuperscript{Asp}(GUU), which may explain why the tRNA can function in translation elongation and why it is imported into mitochondria.

Thus, it is a reasonable hypothesis that eEF1 or the precursor of EF-Tu might be responsible for the observed specificity of mitochondrial tRNA import. However, direct experimental evidence is missing, and it can at present not be excluded that the import specificity is mediated by as yet unknown proteins.

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Localization Determinants in Trypanosomal tRNAs\textsuperscript{Met}


