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The T-Stem Determines the Cytosolic or Mitochondrial Localization of Trypanosomal tRNAs^{Met}

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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 trypanosomatids (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^{Lys} (Entelis *et al*., 1998). In *Tetrahymena*, one of three nucleus-encoded homologuous tRNA^{Gln} isoacceptors, the tRNA^{Gln}(UUG), is imported into mitochondria (Rusconi and Cech, 1996b), and it has been shown that the anticodon of this tRNA is both necessary and sufficient for import (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 tRNA^{Ala} 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 tRNA^{Val} (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^{Glu}(UUC) and tRNA^{Gln}(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-i}) 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-i} 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-i} the extent of import of tRNA^{Met-e} might be mediated by nucleotide modifications.

MATERIALS AND METHODS

Cells

Procyclic *T. brucei*, stock 427, was 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 3.5×10^7 cells/ml and washed once in cold 20 mM sodium phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose. The resulting cellular pellets were either used to isolate total RNA or to prepare mitochondria by digitonin extraction.

Transfection of T. brucei

For expression of the tRNA variants derivatives of the pHD-437 plasmid were used that allow stable integration into the trypanosomal rDNA loci (Biebinger *et al*., 1996). The *Kpn*I/*Bam*HI 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 tRNAs were expressed containing 261 nucleotides of the 5′-flanking region of the tRNA^{Leu}(CAG) gene and their own 3′-flanking region. The constructs were linearized with *Not*I, electroporated into *T. brucei* and transformants were selected with phleomycine and cloned as described previously (Beverley and Clayton, 1993).

Analysis of In Vivo 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 supernatants were 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 guanidiumisothiocyanate method as described previously (Chomczyinski and Sacchi, 1987). Isolated total (0.5×10^7 cell equivalents) and mitochondrial RNA (10^8 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-i}, 5′GTTGGTTTCGATCCAACG3′; wild-type tRNA^{Met-e}, 5′GTGAGGCTCGAACTCACG3′; tRNA^{Met} variants carrying the tagged D-arm of the tRNA^{Met-i}, 5′ CGCTCTTCCCCTGAGCCA3′; tRNA^{Met} variants carrying the tagged D-arm of the tRNA^{Met-e}, 5′CGCTCTGCCGACTGAGCC3′; wild-type tRNA^{Lys}, 5′GTGGCACCCCCCGTGGGGCTCGAACCCA3′; variant tRNA^{Lys}, 5′CAACGTCCTCACGGTTAA3′; wild-type tRNA^{Ile}, 5′TGCTCCCGGCGGGTTCGAA3′; and variant tRNA^{Ile}, 5′CCAACGTCCTTCGGTTCA3′.

Isolation of Native tRNAs^{Met}

To isolate native, fully modified tRNAs, tRNA^{Met-e} and tRNA^{Met-i} present in total RNA fraction (5 μg) and the RNA^{Met-e} present in the mitochondrial RNA fraction (5 μg) were labeled by using the highly efficient and specific 3′-end splint-labeling protocol. In this method, an oligonucleotide (5′AGTGGTGCGATCGGTGAGGCTCGAACTCA 3′ for tRNA^{Met-e} and 5′CAAGTGGTCCTTCCGGCCGGAATCGAAC3′ for tRNA^{Met-i}) is hybridized to the 3′ end of the targeted tRNA where it leaves a 5′ overhang. Sequenase will then add the corresponding complementary nucleotide (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 gels, 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 isotonically isolated mitochondria (200 μg of protein) (Hauser *et al*., 1996). After the addition of 2 pmol of substrate tRNA the reaction was incubated for 10 min at 27°C in either the absence or the presence of a mixture containing 2 mM ATP, 2 mM pyrophosphate, 0.6 mM creatine phosphate, and 0.7 μg of creatine kinase (Roche Diagnostics, 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 27°C. Finally, 1 ml of SoTE containing 4 mM EGTA and 4 mM EDTA was added, the mitochondria were reisolated by centrifugation (20,000 $\times$ *g*/4°C), and the supernatant was removed. The mitochondrial pellet was resuspended in 100 μl of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2% of SDS. RNA was isolated by phenol extraction and ethanol precipitation and analyzed 8 M urea/10% polyacrylamide gels.

Isolation and Direct Sequencing of tRNA^{Met-i}

Separation of tRNAs by two-dimensional (2D)-PAGE and dot blotting were performed according to Maréchal-Drouard *et al*. (1995). Determination of the tRNA sequence was performed using the postlabeling technique described in Stanley and Vassilenko (1978). Briefly, 100 ng of pure tRNA^{Met-i} was randomly hydrolyzed in the presence of 3 μl of formamide for 2 min at 85°C. After lyophilization, the generated tRNA fragments were 5′-end labeled using T4 polynucleotide kinase and separated on a 7 M urea/15% polyacrylamide gel. The gel was analyzed by autoradiography and each band containing a 5′-end–labeled tRNA fragment was cut out and eluted. The eluted tRNA fragments were then digested to completion by using 0.1 μg of nuclease P1. The reaction was performed at 22°C for 16 h in a buffer containing 50 mM ammonium acetate at pH 5.3. Identification of the labeled 5′-end nucleotide of each fragment was achieved by one- or two-dimensional thin layer chromatography (TLC) analysis as described previously (Nishimura, 1979).

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; Kapushoc *et al*., 2000; Tan *et al*., 2002b). Therefore, because the cytosolic tRNA^{Met-i} 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-i} 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 tRNAs^{Met} by oligonucleotide hybridization. The presence of the tag does not alter the localization of the cytosol-specific tRNA^{Met-i} nor that of the imported tRNA^{Met-e} (our unpublished data). All transgenic tRNAs were expressed in the same 5′-genomic context corresponding to the 5′-flanking sequence of the tRNA^{Leu}(CAG) because this sequence allowed efficient expression of all tRNA variants but does not influence their intracellular localization.

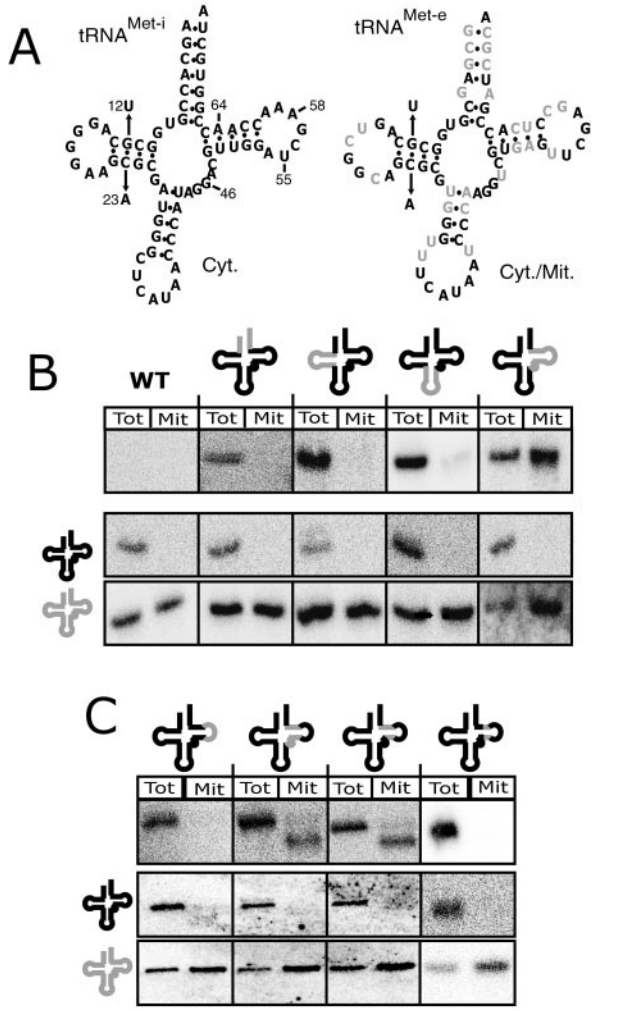

Figure 1. Intracellular localization of $tRNA^{Met\text{-}i}$ variants. (A) Predicted secondary structures of cytosolic $tRNA^{Met\text{-}i}$ and imported $tRNA^{Met\text{-}e}$. Nucleotides that are different between the two molecules are shown in gray in the $tRNA^{Met\text{-}e}$. Numbering of nucleotides is according to Sprinzl *et al*. (1996). The tag (U12:A23) introduced to discriminate the transgenic tRNAs from the corresponding wild-type ones and the in vivo localization of the tRNAs are indicated. (B) Northern blot analysis of wild-type cells (WT) and of cell lines expressing tagged $tRNA^{Met\text{-}i}$ variants containing the acceptor stem, the D-arm, the anticodon stem loop, and the T-arm of the $tRNA^{Met\text{-}e}$. Sequences originating from the $tRNA^{Met\text{-}i}$ are indicated in black; sequences stemming from the $tRNA^{Met\text{-}e}$ are shown in gray throughout the figure. RNA isolated from total cells (Tot.) and from digitonin-extracted mitochondrial fractions (Mit.) was analyzed. Top, Northern blot probed for the tagged transgenic $tRNA^{Met\text{-}i}$ variants. Middle, duplicate blot probed for the cytosol-specific $tRNA^{Met\text{-}i}$. Bottom, duplicate blot probed for wild-type $tRNA^{Met\text{-}e}$. (C) Same as B but cell lines expressing tagged $tRNA^{Met\text{-}i}$ variants containing the T-loop, the T-stem with the variable loop, the T-stem only, or the single T-stem nucleotide pair A52:62U of the $tRNA^{Met\text{-}e}$ were analyzed.

In Vivo Localization of $tRNA^{Met\text{-}i}$ Variants

In a first series of experiments cell lines expressing variants of $tRNA^{Met\text{-}i}$ carrying the acceptor stem, the D-arm, the anticodon stem loop or the T-stem loop (T-arm) of the imported $tRNA^{Met\text{-}e}$ were produced. Total and mitochondrial RNA from the transgenic cell lines where analyzed for the presence of the corresponding $tRNA^{Met\text{-}i}$ variants by using specific oligonucleotide hybridization. Figure 1B shows that the T-arm of the imported $tRNA^{Met\text{-}e}$ is sufficient to confer a mitochondrial localization to the normally cytosol-specific $tRNA^{Met\text{-}i}$. The three other domains of the $tRNA^{Met\text{-}e}$ do not seem to contain targeting information. The bottom two panels of Figure 1B show the intracellular distribution of wild-type $tRNA^{Met\text{-}i}$, which serves as cytosolic marker, and that of wild-type $tRNA^{Met\text{-}e}$, respectively. Comparison of the hybridization signals on the top and bottom panels of the right column of Figure 1B indicate that the $tRNA^{Met\text{-}i}$ variant that carries the T-arm of the $tRNA^{Met\text{-}e}$ is imported with comparable efficiency than the wild-type $tRNA^{Met\text{-}e}$. In a further analysis, we tested whether the whole T-arm is required for targeting of $tRNAs^{Met}$. Four cell lines expressing variants of $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}e}$ 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 $tRNA^{Met\text{-}i}$ and the $tRNA^{Met\text{-}e}$ differ by two nucleotide pairs only (Figure 1A). Further dissection of the T-stem localization determinants shows that the $tRNA^{Met\text{-}i}$ variant carrying the single $tRNA^{Met\text{-}e}$-derived nucleotide pair A52:62U remained in the cytosol (Figure 1C, far right), whereas the $tRNA^{Met\text{-}i}$ variant carrying the $tRNA^{Met\text{-}e}$ nucleotide pair G51:63C was imported, albeit less efficiently than the wild-type $tRNA^{Met\text{-}e}$ (Figure 3B, right) (Note that the $tRNA^{Met\text{-}i}$ variant carrying the G51:C63 nucleotide pair of the $tRNA^{Met\text{-}e}$ is identical to the $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}i}$ variants carrying the T-stem of the $tRNA^{Met\text{-}e}$ 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 $tRNA^{Met\text{-}i}$, 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 $tRNA^{Met\text{-}i}$ variant shown in Figure 3B, right.

Finally, we also did the converse experiments. Variants of the $tRNA^{Met\text{-}e}$ carrying the T-arm or the T-stem only of the cytosolic $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}e}$ that carries the T-stem of the $tRNA^{Met\text{-}i}$ (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 $tRNAs^{Met}$.

In Vivo Localization of Variant $tRNA^{Lys}$ and $tRNA^{Ile}$

Do the T-arm localization determinants of the $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}i}$ (Figure 3A). Cell fractionation and Northern analysis show that both of these molecules are retained in the cytosol (Figure 3, B and C, left column). Furthermore, we also prepared cell lines expressing variant $tRNAs^{Met\text{-}i}$ carrying the T-stem of either the $tRNA^{Lys}$ (as discussed above, this tRNA is identical to the

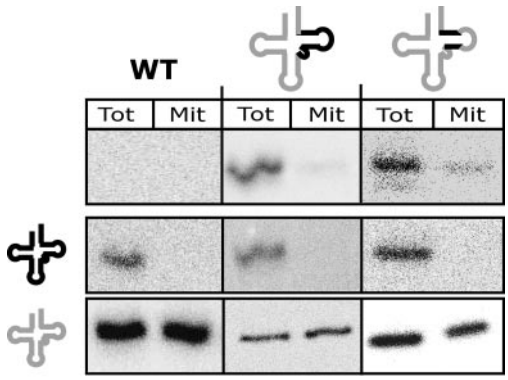

Figure 2. Intracellular localization of tRNA^{Met-e} variants. Northern blot analysis of wild-type cells (WT) and of cell lines expressing tagged tRNA^{Met-e} containing the T-arm or the T-stem only of the tRNA^{Met-i}. The panels are arranged as in Figure 1.

tRNA^{Met-i} variant carrying the G51:C63 of the tRNA^{Met-e}) or the tRNA^{Ile} and showed that both of these were imported into mitochondria. However, whereas the T-stem of the tRNA^{Lys} induced import whose extent was comparable with that of wild-type tRNA^{Lys} (Figure 3B, right column), the tRNA^{Met-i} containing the T-stem of the tRNA^{Ile} was less efficiently imported than wild-type tRNA^{Ile} (Figure 3C, right column).

Localization of tRNA^{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^{Glu}(UUC) and tRNA^{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^{Met-i}, we determined the nucleotide modification in the T-arm region of *T. brucei* wild-type tRNA^{Met-i}. To that end, the tRNA^{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^{Met-i}. Indeed, the pseudouridine probably occurs in all tRNAs (Sprinzl *et al*., 1996), and a m(1)A at position 58 has been detected in many different tRNAs, including the tRNA^{Met-i} and tRNA^{Met-e} (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^{Met-i} will have any effect on the modification pattern of the tRNA^{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^{Met-i} carrying an A-to-U

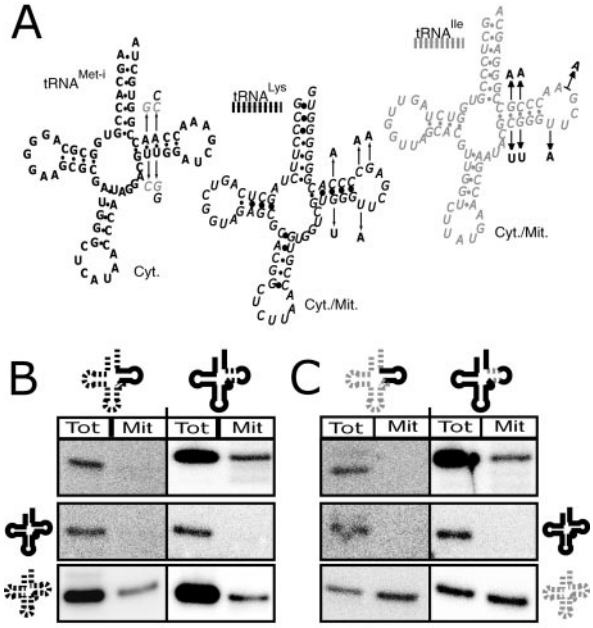

Figure 3. Intracellular localization of tRNA^{Lys} and tRNA^{Ile} variants. (A) Predicted secondary structures of cytosolic tRNA^{Met-i}, imported tRNA^{Lys} and tRNA^{Ile}. The in vivo localization of the wild-type tRNAs is indicated. Sequences originating from the tRNA^{Met-i} are indicated in black. Sequences stemming from the tRNA^{Lys} are depicted in black italic letters in A and by black broken lines in B. tRNA^{Ile} is shown in gray italic letters in A and by gray broken lines in C. The nucleotide changes required to transform the T-arm of the tRNA^{Met-i} into the one of tRNA^{Lys} and tRNA^{Ile} are indicated. The same is done for the changes required to transform the T-arm of the tRNA^{Lys} or tRNA^{Ile} into the T-arm of the tRNA^{Met-i}. (B) Northern blot analysis of total (Tot.) and mitochondrial (Mit.) RNA from cell lines expressing a tRNA^{Lys} variant containing the T-arm of the tRNA^{Met-i} and a tagged tRNA^{Met-i} variant containing T-arm of the tRNA^{Lys} (note that this tRNA is identical in sequence to a tRNA^{Met-i} variant carrying the G51:C63 of the tRNA^{Met-e}). Top, Northern blot probed for the transgenic tRNA^{Lys} and tRNA^{Met-i} variants, respectively. Middle, duplicate blot probed for the cytosol-specific tRNA^{Met-i}. Bottom, duplicate blot probed for wild-type tRNA^{Lys}. (C) Same as B but cell lines expressing a tRNA^{Ile} variant containing the T-arm of the tRNA^{Met-i} and a tagged tRNA^{Met-i} variant containing T-arm of the tRNA^{Ile} were analyzed. Bottom, duplicate blot probed for wild-type tRNA^{Ile}.

replacement at position 58. The transgenic tRNA^{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^{Met-i} of yeast and plants and consists of an unusual 2′-*O*-phosphoribosyl purine [R(p)] at position 64. The function of Rp(p) is to prevent binding of the tRNA^{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^{Met-i} containing the inversed base pair 50A:64U instead of the wild-type 50U:64A. This tRNA^{Met-i} variant lacks any putative R(p) modification but nevertheless remains cytosol specific (Figure 4D, right).

In Vitro Import of Isolated tRNA^{Met}

Analysis of transgenic trypanosomes expressing different tRNA^{Met} variants allowed to identify the two adjacent un-

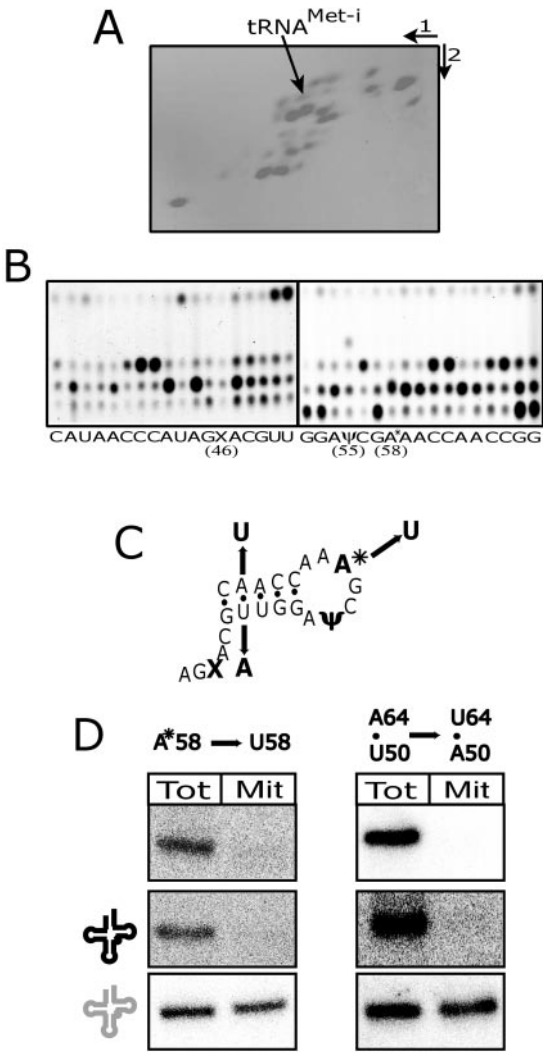

Figure 4. Localization of trypanosomal tRNA^{Met-i} does not require specific nucleotide modifications. (A) Separation of *T. brucei* total tRNAs by 2D-PAGE. The spot identified by dot blot hybridization using a 5′-end–labeled oligonucleotide specific to tRNA^{Met-i} as a probe is indicated by the arrow. (B) Determination of the sequence of the tRNA^{Met-i} (position 34 to position 68) by using the method described by Stanley and Vassilenko (1978). Modified nucleotides were detected at position 55 (ψ) and at position 58 (A*). Both were analyzed by 2D-TLC (our unpublished data): position 55 was shown to correspond to pseudouridine, whereas position 68 most likely corresponds to a 1-methyladenosine derivative [m(1)A]. The nucleotide at position 46 in the variable loop (indicated by X) could not be determined. (C) Predicted secondary structure of the T-arm of tRNA^{Met-i} showing the position of the modified nucleotides. The A-to-U replacement introduced to abolish the m(1)A derivative at position 58, and the 50U:64A to A50:U64 nucleotide pair switch, which were introduced in the respective tRNA^{Met-i} gene of the transgenic cell lines are indicated. (D) Northern blot analysis of total (Tot.) and mitochondrial RNA (Mit.) of transgenic cell lines expressing tagged tRNA^{Met-i} variants containing the nucleotide replacements show in C. The panels are arranged as in Figure 1.

modified T-stem nucleotide pairs (51:63 and 52:62) as the determinants for in vivo localization. In a next step, we tested whether tRNA^{Met} variants that are in vivo either cytosolic or in part mitochondrially localized can be imported into isolated mitochondria. Preliminary experiments

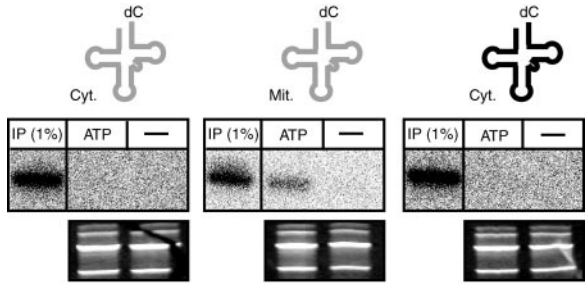

Figure 5. In vitro import of isolated tRNAs^{Met} into mitochondria of *T. brucei.* In vitro import assays in the presence and absence of ATP using isolated 3′-splint–labeled tRNAs^{Met}. Left, tRNA^{Met-e} isolated from cytosolic RNA (Cyt.). Middle, tRNA^{Met-e} isolated from mitochondrial RNA (Mit.). Right, tRNA^{Met-i} isolated from cytosolic RNA (Cyt.). Due to the splint labeling procedure all tRNAs substrates carry a 3′ dCTP. All samples have been treated with micrococcus nuclease. IP (1%), 1% of the added substrates. Bottom, loading controls showing ethidium bromide-stained region of the same gel. The two bands correspond to the two mitochondrial rRNAs.

showed that in vitro transcripts of all tRNAs^{Met} were imported (our unpublished data). Import of in vitro transcribed tRNA^{Met-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 tRNA^{Met-i} is due to the lack of nucleotide modifications in the in vitro transcript because the in vivo localization determinants of tRNA^{Met-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 tRNAs^{Met} were used for in vitro import, the obtained results are compatible with the in vivo analysis. No import was observed for either the tRNA^{Met-i} or the tRNA^{Met-e} isolated from cytosolic RNA, whereas the tRNA^{Met-e} isolated from the mitochondrial fraction was imported (Figure 5). Because the cytosolic and the mitochondrial tRNA^{Met-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 aminoacyl-tRNA synthetases are not involved in import, because an intron-containing tRNA^{Tyr} that cannot be aminoacylated 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^{Gln}(CUG) containing the D-arm of the imported tRNA^{Ile} 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^{Ile} variant containing the D-arm of the cytosolic tRNA^{Gln}(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^{Lys} and tRNA^{Ile}. The tRNA^{Ile} is the same tRNA whose mitochondrial localization in *L. tarentolae* could not be changed by transplantation of the D-arm of the cytosolic tRNA^{Gln}(CUG) (Lima and Simpson, 1996). Interestingly, the nucleotide pair U51:A63 is essentially unique for the cytosolic tRNA^{Met-i} and is only found in one, the tRNA^{Asn}(GUU), of the 40 known trypanosomal elongator tRNAs (Figure 6). tRNA^{Asn}(GUU) is in part imported into mitochondria, indicating that in this case the T-stem localization determinants are not functional.

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 anticodon UUG of the imported tRNA^{Gln} is both necessary and sufficient to induce import of any of the three tRNA^{Gln} isoacceptors (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 anticodon 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 Maréchal-Drouard, 2000), the in vivo localization determinants are not conserved between different species.

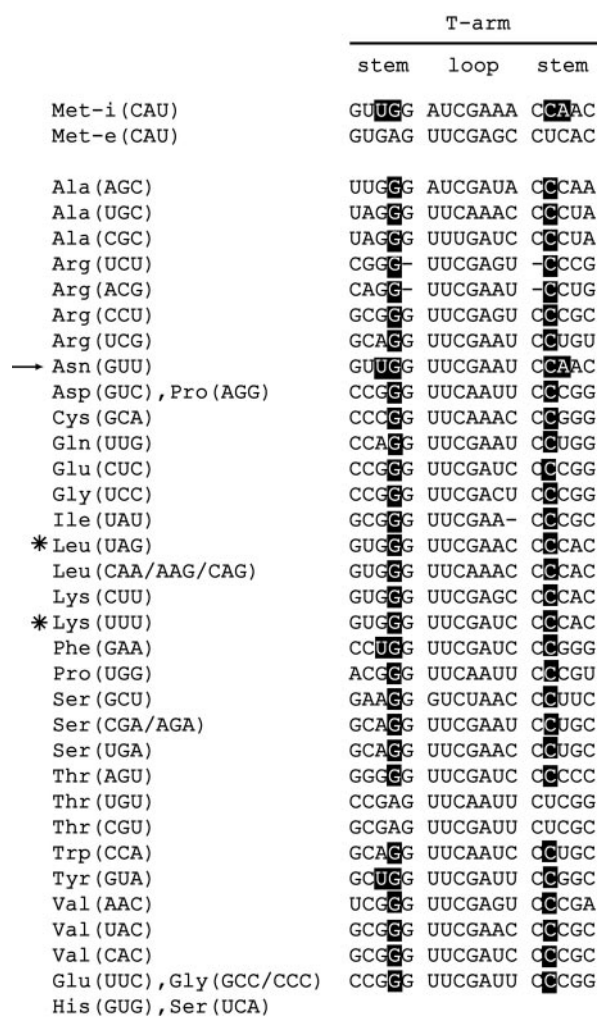

Figure 6. Alignments of T-arm sequences of all known *T. brucei* tRNAs. tRNA^{Met-i} and tRNA^{Met-e} are listed at the top. tRNA^{Ile} and tRNA^{Lys}, which were used in the in vivo experiments shown in Figure 3, are indicated by the asterisks. Sequences were taken from http://zoosun00.unifr.ch/Trypanos/WWW%20tRNA/tRNAGenOrg.html. The tRNA^{Met-i} 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-i}, are boxed. With the exception of the tRNA^{Asn} (indicated by the arrow) the two nucleotide pairs (U51:A63 and G52:C62) occur only in the tRNA^{Met-i}.

In Vitro Import Experiments

The specificity of mitochondrial tRNA import in trypanosomatids has also been investigated in reconstituted systems. As illustrated below, the results were in many respects different from the ones obtained in vivo.

In vitro import of tRNA^{Leu}(CAA) into mitochondria of *T. brucei* requires a 5′ extension (Yermovsky-Kammerer and Hajduk, 1999), whereas in vivo experiments showed that the 5′-flanking sequence of the same tRNA can be replaced without affecting import (Tan *et al*., 2002b).

In *Leishmania*, a short sequence element in the D-arm has been proposed as an import signal for the tRNA^{Tyr} (Mahapatra *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 $tRNA^{Met}$ 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 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 $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}i}$ is not (Tan *et al*., 2002b). Could the import specificity therefore be linked to the initiation or elongation function of tRNAs? For a $tRNA^{Met\text{-}i}$ 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 $tRNA^{Met\text{-}i}$ act as a retention signal, it could be the interaction of the $tRNA^{Met\text{-}i}$ with the eIF2 that prevents import. We believe that this is not very likely because a $tRNA^{Met\text{-}i}$ variant carrying the acceptor stem of the $tRNA^{Met\text{-}e}$ remains in the cytosol (Figure 1B). The A1:U72 nucleotide pair of the acceptor stem, however, is a specific hallmark of eukaryotic $tRNA^{Met\text{-}i}$ 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 the EF-Tu. The main determinants responsible for preventing the binding of vertebrate $tRNA^{Met\text{-}i}$ 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 $tRNA^{Asn}$(GUU) contains the cytosolic T-arm localization determinants and is therefore predicted not bind to eEF1. Nevertheless, it is imported into mitochondria and clearly functions in translation elongation (it is the only known $tRNA^{Asn}$ in *T. brucei*) (Tan *et al*., 2002b). This suggests that the antideterminants for eEF1 binding are masked by some unknown structural feature specific for the $tRNA^{Asn}$(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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