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Sequence-dependent in vivo importation of tRNAs into the mitochondrion of *Leishmania tarentolae*

BEATRIZ D. LIMA[1,2] and LARRY SIMPSON[2,3,4]

[1] Departamento de Biologia Celular, Universidade de Brasilia, CEP 70910-900, Brazil
[2] Department of Molecular, Cellular and Developmental Biology, University of California, Los Angeles, California 90095-1662, USA
[3] Department of Medical Microbiology and Immunology, University of California, Los Angeles, California 90095-1662, USA
[4] Howard Hughes Medical Institute, University of California, Los Angeles, California 90095-1662, USA

ABSTRACT

Sequence determinants for the importation of tRNAs into the mitochondrion of *Leishmania tarentolae* in vivo were investigated. tRNA^{Ile}(UAU) is exclusively localized within the mitochondrion and tRNA^{Gln}(CUG) exclusively in the cytosol (Lye LF, Chen DHT, Suyama Y, 1993, *Mol Biochem Parasitol 58*:233-246; Shi X, Chen DHT, Suyama Y, 1994, *Mol Biochem Parasitol 65*:23-37). *L. tarentolae* cells were transfected with plasmids encoding either tRNA^{Ile} or tRNA^{Gln} that were tagged with altered sequences in the D loop, permitting discrimination from the endogenous tRNAs. Primer extension analysis was used to show that the plasmid-encoded genes were expressed and that the tagged tRNAs showed a similar intracellular localization as the endogenous tRNAs. Exchange or deletion of the 5′-flanking genomic sequences had no effect on the expression or mitochondrial localization of the tagged tRNA^{Ile} or on the expression or cytosolic localization of the tagged tRNA^{Gln}, suggesting that the signals for importation are localized within the tRNA itself. Swapping the D loop+stem from the exclusively cytosolic tRNA^{Gln} with that from the tRNA^{Ile} produced a partial mitochondrial localization of the plasmid-expressed mutated tRNA^{Gln}. However, D loop exchange did not eliminate the mitochondrial localization of the plasmid-expressed mutated tRNA^{Ile}, suggesting that tertiary structure or additional sequence elements may be involved in the importation signal.

Keywords: in vivo importation; *Leishmania*; transfection; tRNA

INTRODUCTION

Most evidence for RNA importation into mitochondria is indirect: the genes for many mitochondrial tRNAs (mito-tRNAs) are absent from the mitochondrial genomes of several eukaryotes and are located within their nuclear genomes. For example, the mito-tRNA$_2^{Lys}$(CUU) in *Saccharomyces cerevisiae* (Martin et al., 1979; Tarassov & Entelis, 1992), two mito-tRNAs in cnidarians (Wolstenholme, 1992), 19 mito-tRNAs in *Chlamydomonas reinhardtii* (Boer & Gray, 1988), 19 mito-tRNAs in *Paramecium aurelia* (Pritchard et al., 1990), 26 mito-tRNAs in *Tetrahymena pyriformis* (Suyama, 1986), all mito-tRNAs in *Plasmodium falciparum* (Feagin, 1992), *Trypanosoma brucei* (Hancock & Hajduk, 1990), and *Leishmania tarentolae* (Simpson et al., 1989), and a few in higher plants (Small et al., 1992) are all nuclear-encoded. There are also two additional candidates for RNA importation into mitochondria: the site-specific endonuclease involved in mitochondrial RNA primer processing (RNAse MRP) (Li et al., 1994), and the RNA component of mammalian mitochondrial RNAse P (G. Attardi, pers. comm.).

Direct experimental evidence for importation in plant mitochondria has been obtained by producing a transgenic potato plant carrying a leucine tRNA gene from bean nuclear DNA and showing that the expressed bean tRNA^{Leu} fractionated with mitochondria (Small et al., 1992).

Evidence for importation of the tRNA^{Lys}(CUU) in *S. cerevisiae* was obtained by showing that this tRNA was found in a mitochondrial fraction within 1-2 h after it was introduced into yeast cells by electroporation (Tarassov & Entelis, 1992). Additional evidence for importation of tRNAs into yeast mitochondria involved the establishment of an in vitro importation assay using nuclease-protection of labeled tRNA incubated with purified mitochondria. The in vitro importation of the tRNA^{Lys}(CUU) required mitochondria, ATP, and a cytosolic S-100 fraction, the active components of which

Reprint requests to: Larry Simpson, 6780 MacDonald Building, Howard Hughes Medical Institute, UCLA, Los Angeles, California 90095-1662, USA; e-mail: simpson@sunls.hhmi.ucla.edu.

included the cytosolic tRNA synthetases (Tarassov & Entelis, 1992; Tarassov et al., 1995a). A requirement for an intact protein translocating system for the in vitro importation of $tRNA^{Lys}$ has also been demonstrated (Tarassov et al., 1995b).

In the trypanosomatid protozoa, *L. tarentolae* and *T. brucei*, no tRNA gene sequences are encoded by the mitochondrial maxicircle genome, and mito-tRNAs were found to hybridize to nuclear DNA (Simpson et al., 1989; Hancock & Hajduk, 1990; Mottram et al., 1991). Most of the mito-tRNAs of *L. tarentolae* comigrated during two-dimensional gel electrophoresis with cytosolic tRNAs, but a subset appeared to be unique to the organelle (Simpson et al., 1989). A total of 13 *L. tarentolae* nuclear-encoded tRNA genes have been sequenced to date (Lye et al., 1993; Shi et al., 1994). By northern blot analysis, several tRNAs were found to be localized mainly in the mitochondrion, one tRNA was found exclusively in the cytosol, and others distributed to different extents between both compartments (Lye et al., 1993; Chen et al., 1994; Shi et al., 1994). For example, $tRNA^{Ile}$(UAU) from *L. tarentolae* was detected mainly within a mitochondrial fraction, and the $tRNA^{Gln}$(CUG) was exclusively in a cytosolic fraction (Lye et al., 1993; Shi et al., 1994). However, the isoacceptor $tRNA^{Gln}$(UUG) showed partial mitochondrial localization (Lye et al., 1993; Shi et al., 1994).

Transfection of a plasmid-encoded $tRNA^{Thr}$(AGU) into *L. tarentolae* cells was used to demonstrate that this expressed tRNA was partially mitochondrial-localized, and a four-nucleotide insertion in the tRNA variable loop was found to completely eliminate mitochondrial localization (Chen et al., 1994).

Stable transfection was also used in *T. brucei* to show that an intron-containing $tRNA^{Tyr}$ gene expressed a tagged tRNA that became localized within the mitochondrion (Schneider et al., 1994a, 1994b). Hancock et al. (1992) and Hancock and Hajduk (1992) have shown previously in *T. brucei* that 5′-extended tRNAs were found in a purified mitochondrial fraction in addition to mature tRNAs, and that these 5′-leader sequences could be processed by heterologous or homologous RNase P-like activities. The authors suggested that these 5′-extended tRNAs could be the substrates for importation into the organelle. Hauser and Schneider (1995) showed recently, by transfection experiments, that 5′ flanking sequences are not involved in mitochondrial localization in vivo of either the homologous intron-containing $tRNA^{Tyr}$ or two heterologous cytosolic tRNAs ($tRNA^{His}$ and $tRNA^{Lys}$), suggesting that the tRNA structure itself is sufficient to specify mitochondrial targeting.

Apparent importation of small RNAs in vitro has been reported using a mitochondrial fraction from *Leishmania donovani* (Mahapatra et al., 1994): a synthetic antisense transcript from the 5′ upstream region of the β-tubulin gene became RNase-resistant after incubation with isolated mitochondria. This apparent importation was dependent on ATP and was competed by homologous tRNA and small rRNAs, and did not require a soluble cytosolic fraction.

In this paper, we employ plasmid transfection techniques to express a mitochondrial-localized $tRNA^{Ile}$(UAU) and a cytosolic-localized $tRNA^{Gln}$(CUG) in *L. tarentolae*, and we have investigated the sequence determinants for in vivo targeting to the mitochondrion.

RESULTS

Transformation of cells with plasmids encoding tagged $tRNA^{Ile}$ and tagged $tRNA^{Gln}$

Various constructs of $tRNA^{Ile}$(UAU) and $tRNA^{Gln}$(CUG) genes were tagged with nucleotide changes in the D loop+stem to allow us to distinguish these tRNAs from the endogenous tRNAs. Figure 1A shows the gene sequences for wild-type (wt) $tRNA^{Ile}$(UAU) and $tRNA^{Gln}$(CUG), and the respective tagged and mutated tRNA genes, $tRNA^{Ile}$(D-tag), $tRNA^{Gln}$(D-tag), $tRNA^{Ile}$(D-Gln), and $tRNA^{Gln}$(D-Ile). The modified tRNA genes used in the experiments are presented in Figure 1C. Creation of these constructs involved swapping the 5′-flanking sequences between the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes (shown in Fig. 1B), deletion of the 5′-flanking sequences, and swapping of the D loop+stem sequences between the two tRNA genes. The 37-bp 3′-flanking sequence of the $tRNA^{Ile}$ gene (Fig. 1B) was used for all constructs to provide a putative termination signal for pol III transcription.

These gene constructs were cloned in a pUC18 plasmid, into which was inserted a fragment from the pX vector (LeBowitz et al., 1990) containing the selectable marker for neomycin resistance. The final plasmid is shown in Figure 1C. *L. tarentolae* cells were transfected and transformants selected for G418 resistance. Total DNA was isolated from the transformants, and also from pX-transfected cells, digested with *Eco*R I, gel-fractionated, blotted, and hybridized with labeled pX DNA. The strong hybridization signal in all transformants suggests that the plasmids are in high copy number (data not shown).

Expression of the tRNAs and localization in cytosolic or mitochondrial fractions from the various transformants were assayed by primer extension, using specific primers complementary to the anticodon loop/stem region. Specific labeled deoxynucleotides and dideoxynucleotides were used to obtain differential extension and labeling for the endogenous and tagged tRNAs as shown in Table 1.

The purity of the mitochondrial preparations was monitored by measuring the ratio of the three ethidium bromide-stained cytosolic ribosomal RNA (rRNA) bands to the mitochondrial 9S and 12S rRNA bands in a 1% formaldehyde-agarose gel (data not shown), and

A

		D loop	Anticodon loop	Variable loop	TψC loop
$tRNA^{Ile}$	5′	GCTCCCGTGGTCTAGTTGGTTAGGACGCTGGTCTTATGAACCGGATGTCGCGGGTTCGAGCCCCGCCGGGAGCA			3′
$tRNA^{Ile}$(D-tag)	5′	.....CAC.....			3′
$tRNA^{Ile}$(D-Gln)	5′	..........G...C .....TC.....			3′

		D loop	Anticodon loop	Variable loop	TψC loop
$tRNA^{Gln}$	5′	GCTCCTATAGTGTAGC GGTTATCACCTCGGACTCTGAATCCGATAACCCTGGTTCGAGTCCAGGTAGGAGTG			3′
$tRNA^{Gln}$(D-tag)	5′	....AAC.....			3′
$tRNA^{Gln}$(D-Ile)	5′	..........C...TT.....GG.....			3′

B

5' Ile	5′	TCACACGAACTTGGGTCTCCAGGGCGTTCACAACTCCCCACCTACATCCATATTCAGTATGTACCAC 3′
5' Gln	5′	GTTATGGGCTAACCGGGGGGGGGGGGCTCCAGTTATACTGTCACCTTCATCATCCTTGTATGATCAGGC 3′
3' Ile	5′	CTTTTCCGCCCCAAAATCCCCAACACGGAAAAAACCC 3′

C

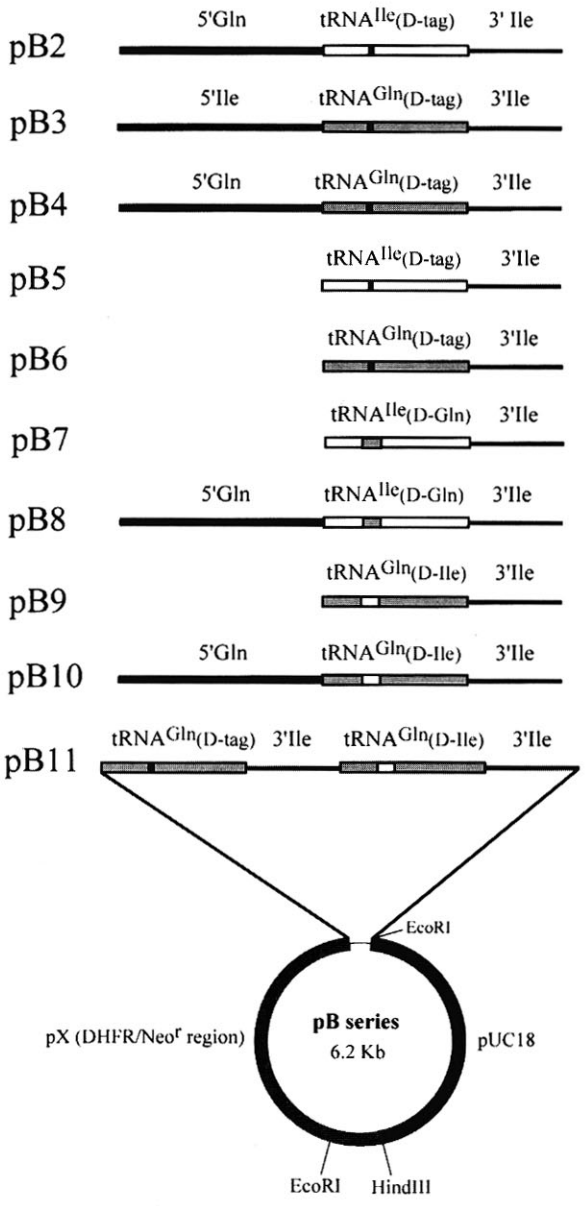

FIGURE 1. Sequences of the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes, 5′ and 3′-flanking regions, and gene constructs. **A:** Nucleotide sequence of *L. tarentolae* $tRNA^{Ile}$(UAU) and $tRNA^{Gln}$(CUG) genes and the corresponding mutants: $tRNA^{Ile}$(D-tag), $tRNA^{Ile}$(D-Gln), $tRNA^{Gln}$(D-tag), and $tRNA^{Gln}$(D-Ile). Dots indicate conserved bases between the mutant and the wild type. tRNA loops are indicated with bars. $tRNA^{Ile}$(D-tag) and $tRNA^{Gln}$(D-tag) contain three point mutations in the D loop that were used as a tagged sequence in the primer extension experiments. $tRNA^{Ile}$(D-Gln) contains a deletion of a T residue and four point mutations in the D loop+stem, becoming a $tRNA^{Gln}$ D loop+stem. $tRNA^{Gln}$(D-Ile) contains an insertion of a T base and four point mutations in the D loop+stem, becoming a $tRNA^{Ile}$ D loop+stem. The point mutations (TC) in the $tRNA^{Ile}$(D-Gln) and (GG) in the $tRNA^{Gln}$(D-Ile) were used as a tagged sequence in the primer extension experiments. **B:** Nucleotide sequence of *L. tarentolae* 5′-flanking region of the $tRNA^{Ile}$(UAU) and of the $tRNA^{Gln}$(CUG) and 3′-flanking region of the $tRNA^{Ile}$(UAU) used in the gene constructs. **C:** Schematic diagrams of the tRNA gene constructs, pB2-pB11. A diagram of the entire pB plasmid is also shown, with an indication of the tRNA inserts and the 3.2-kb fragment from the pX vector containing the neomycin resistance gene.

TABLE 1. Primer extension strategies to discriminate between the endogenous $tRNA^{Ile}$ and $tRNA^{Gln}$ and the corresponding mutated tRNAs expressed from the transfected plasmids.[a]

Assay	$tRNA^{Ile}$	$tRNA^{Ile}$(D-tag)	$tRNA^{Ile}$(D-Gln)	$tRNA^{Gln}$	$tRNA^{Gln}$(D-tag)	$tRNA^{Gln}$(D-Ile)
# 1 (α^{32}P)dTTP						
ddATP	.GGUUAGGACGCUGG..3′	.GGCACGGACGCUGG..3′	.GGUUAUCACGCUGG..3′	.GGUUAUCACCUCGG..3′	.GGAACUCACCUCGG..3′	.GGUUAGGACCUCGG..3′
dCTP	atccTGCGACC..5′	gccTGCGACC..5′	gTGCGACC..5′	gTGGAGCC..5′	gTGGAGCC..5′	atccTGGAGCC..5′
ddGTP	(25 nt)					(25 nt)
				.GGUUAUCACCUCGG..3′	.GGAACUCACCUCGG..3′	
				atAGTGGAGCC..5′	gAGTGGAGCC..5′	
				(23 nt)*	*	
# 2 (α^{32}P)dGTP	.GGUUAGGACGCUGG..3′	.GGCACGGACGCUGG..3′				
ddTTP	tccTGCGACC..5′	tgccTGCGACC..5′				
dCTP		(25 nt)				
# 3 (α^{32}P)dGTP				.GGUUAUCACCUCGG..3′	.GGAACUCACCUCGG..3′	.GGUUAGGACCUCGG..3′
dATP				tagTGGAGCC..5′	tgagTGGAGCC..5′	tccTGGAGCC..5′
ddTTP				(24 nt)	(25 nt)	
ddCTP						
# 4 (α^{32}P)dGTP	.GGUUAGGACGCUGG..3′		.GGUUAUCACGCUGG..3′	.GGUUAUCACCUCGG..3′	.GGAACUCACCUCGG..3′	.GGUUAGGACCUCGG..3′
ddATP	cTGCGACC..5′		agTGCGACC..5′	agTGGAGCC..5′	agTGGAGCC..5′	cTGGAGCC..5′
ddCTP			(23 nt)	(23 nt)	(23 nt)	
ddTTP						
				.GGUUAUCACCUCGG..3′	.GGAACUCACCUCGG..3′	
				tAGTGGAGCC..5′	tgAGTGGAGCC..5′	
				*	(23 nt)*	

[a] Underlined bases indicate mutations. Bases in lower case are those added by reverse transcription to the 3′ end of the primers. Bases in **bold lower case** are radioactively labeled. S1761 primer for $tRNA^{Ile}$, $tRNA^{Ile}$(D-tag), and $tRNA^{Ile}$(D-Gln), and S1762 and S2118 primers for $tRNA^{Gln}$, $tRNA^{Gln}$(D-tag), and $tRNA^{Gln}$(D-Ile) are 21 nt. *, S2118 primer.

also by northern analysis of the extent of contamination of the mito-RNA preparations with cytosolic 18S rRNA. In general, a level of cytosolic rRNA contamination of less than 10% was considered suitable for the primer extension analysis, and most mitochondrial preparations contained less than 5% contamination.

Expression and localization of tagged $tRNA^{Ile}$

Assay #1 distinguishes between wt $tRNA^{Ile}$ and $tRNA^{Ile}$(D-tag), yielding a 25-nt labeled product only with wt tRNA and not with the tagged tRNA (Table 1). The results in Figure 2 show that the endogenous $tRNA^{Ile}$ in wt and pX-transformed cells is mainly localized in the mitochondrial fraction, as reported previously (Shi et al., 1994). There is a decrease in the amount of wt $tRNA^{Ile}$ localized within the mitochondrial fractions in the pB2 and pB5 transformants (Fig. 2), which we speculate may possibly represent overexpression of the tagged tRNAs from the plasmids and subsequent saturation of the importation machinery.

Assay #2 is specific for the $tRNA^{Ile}$(D-tag), yielding a labeled 25-nt product (Table 1). The specificity of the assay for the tagged tRNA is shown by the absence of this product in cytosolic and mitochondrial RNA from wt cells (data not shown) or from cells transformed with pX alone (Fig. 2). The extent of cytosolic rRNA contamination of the mito-RNA preparations from the pB2 and pB5 transformants was minimal (2.6 and 2.3%) and the presence of labeled 25-nt products in both cytosolic and mitochondrial lanes (Fig. 2) indicates a partial mitochondrial localization of the plasmid-expressed tagged $tRNA^{Ile}$.

Because the pB2 plasmid has undergone an exchange of the 5′-flanking sequence with that from the $tRNA^{Gln}$ and the pB5 plasmid has suffered a deletion of the 5′-flanking sequence (Fig. 1C), the results in Figure 2 indicate that exchange or deletion of the 5′-flanking sequence have no effect on either expression or mitochondrial targeting of the tagged tRNA. These results strongly suggest that the mitochondrial targeting signals for the plasmid-expressed $tRNA^{Ile}$(D-tag) must be localized within the RNA molecule itself.

Expression and cytosolic localization of tagged $tRNA^{Gln}$

Primer extension assay #3 distinguishes between wt $tRNA^{Gln}$ and $tRNA^{Gln}$(D-tag) by producing 24-nt or 25-nt labeled products, respectively (Table 1). The presence of a 24-nt wt tRNA band in both cytosolic and mitochondrial RNA lanes for all four transformants (pX, pB6, pB3, and pB4; Fig. 3) is a result of a simultaneous detection of an isoacceptor $tRNA^{Gln}$(UUG), which is known to distribute equally between the two subcellular compartments and which cannot be distinguished by this extension assay (Shi et al., 1994). Two other primers were also tested, but were not able to distinguish the isoacceptors (data not shown).

Figure 3 shows that the $tRNA^{Gln}$(D-tag) gene was expressed in the pB3, pB4, and pB6 transformants due to the presence of a 25-nt labeled product in cyto-tRNA lanes and the absence of this band in the pX transformant. However, this band is present only in the cytosolic RNA preparations and is not detectable in the mito-RNA preparations, indicating a specific cytosolic localization of this plasmid-expressed tagged tRNA in all three transformants.

Figure 3 also shows that the exchange of the 5′-flanking sequence of the $tRNA^{Gln}$(D-tag) with that from the $tRNA^{Ile}$ in the pB3 plasmid and the deletion of the 5′-flanking sequence in the pB6 plasmid had no apparent effect on the expression or cytosolic localization of the tagged tRNAs. These results again indicate that the 5′-flanking sequence of the tRNA gene is not required for expression from a plasmid, and that mitochondrial targeting signals reside within the tRNA itself. These targeting signals are apparently absent in

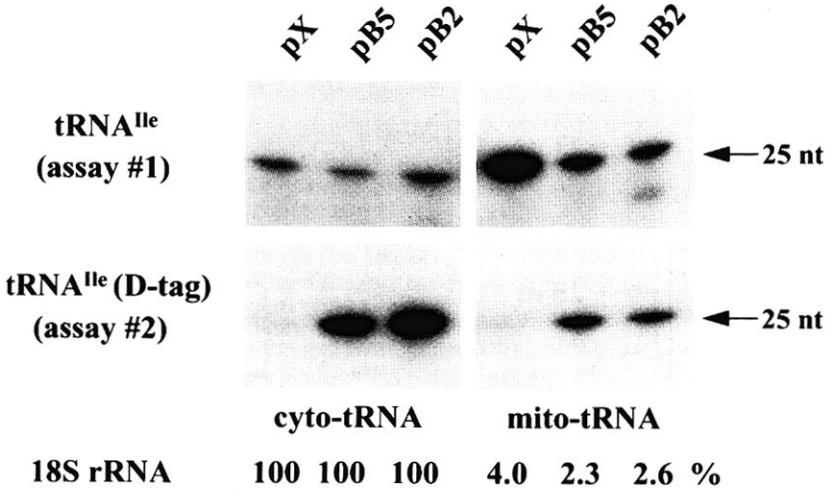

FIGURE 2. Expression and mitochondrial localization of the $tRNA^{Ile}$(D-tag). Cyto-tRNA and mito-tRNA from the pX, pB2, and pB5 transformants were analyzed by using primer extension assays #1 and #2. Sizes of the expected extension products are indicated on the right. The pB5 construct does not contain any 5′-flanking region, and the pB2 construct contains the 5′-flanking region of the $tRNA^{Gln}$. Extent of contamination of cytosolic 18S rRNA is indicated.

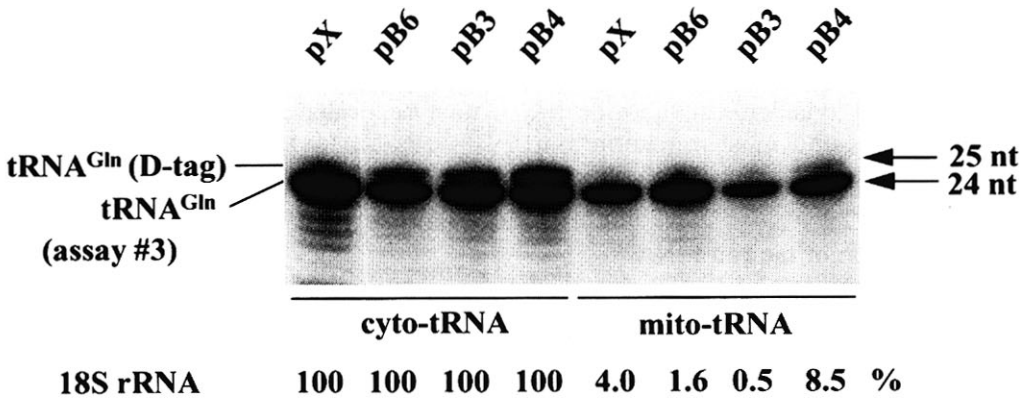

FIGURE 3. Expression and cytosolic localization of tRNA^{Gln}(D-tag). Cyto-tRNA and mito-tRNA from pX, pB3, pB4, and pB6 transformants were analyzed by using primer extension assay #3. Sizes of the expected extension products are indicated on the right. The pB3 construct contains the gene for tRNA^{Gln}(D-tag) with the 5′-flanking region of tRNA^{Ile}. The pB4 construct has the tRNA^{Gln}(D-tag) with the 5′-flanking region of the tRNA^{Gln}, and the pB6 construct lacks any 5′-flanking region. Extent of contamination of cytosolic 18S rRNA is indicated.

the expressed tagged tRNA^{Gln}, leading to an exclusive cytosolic localization.

Swapping the D loop+stem between the tRNA^{Ile} and tRNA^{Gln} produces a mitochondrial localization of tRNA^{Gln}(D-Ile)

The creation of mitochondrial targeting by mutating a tRNA that normally is only cytosolic-localized would provide information about specific targeting signals. To investigate the effect of specific sequence changes on the localization of tRNA^{Gln}, the plasmids pB9 and pB10 were constructed, in which the D loop+stem from the tRNA^{Ile} was substituted for the endogenous D loop+stem of the tRNA^{Gln}. In addition, the pB10 plasmid contained the tRNA^{Gln} 5′-flanking sequence and the pB9 plasmid lacked this sequence. Primer extension assay #1 (Table 1) produces a 25-nt labeled product only with the tRNA^{Gln}(D-Ile) and not with wt tRNA^{Gln}. As shown in Figure 4, the pB9 and pB10 transformants both show the presence of tRNA^{Gln}(D-Ile) in the mitochondrial fraction, with the amount being approximately equivalent to that of the isoacceptor tRNA^{Gln}(UUG). The low levels of cytosolic rRNA in these mito-tRNA preparations (1.2 and 1.7%) confirm that the detection of tRNA^{Gln}(D-Ile) in the mito-tRNA fraction is not due to cytosolic contamination. These results show that replacement of the tRNA^{Gln} D loop with the tRNA^{Ile} D loop produces a change in the subcellular compartmentalization of the expressed tRNA, resulting in a partial mitochondrial targeting. This suggests the presence of specific targeting signals localized within the tRNA^{Ile} D loop+stem.

The converse experiment is shown in Figure 5, in which the tRNA^{Gln} D loop+stem replaced the tRNA^{Ile} D loop+stem in plasmids pB7 and pB8. Assay #4 yields

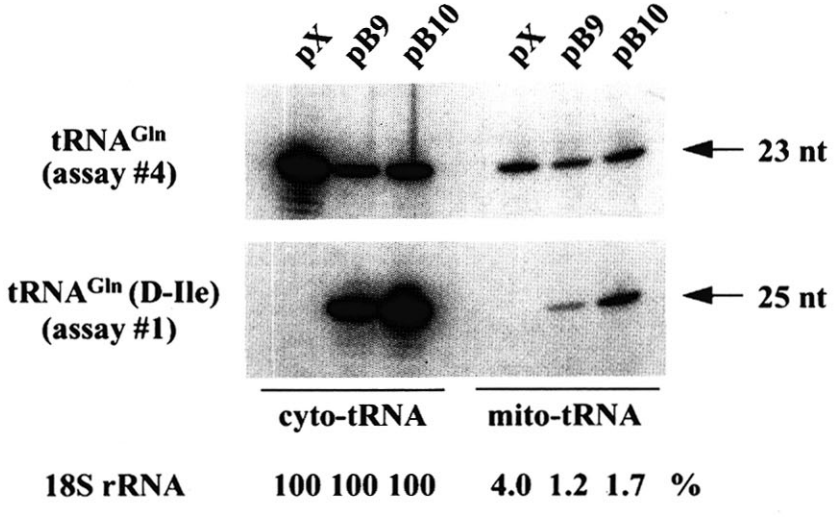

FIGURE 4. Expression and localization of the tRNA^{Gln}(D-Ile). Cyto-tRNA and mito-tRNA from pX, pB9, and pB10 transformants were analyzed by using primer extension assays #1 and #4. Sizes of the expected extension products are indicated on the right. The pB9 construct contains the gene for tRNA^{Gln}(D-Ile) without the 5′-flanking region, and the pB10 construct contains the same gene with the 5′-flanking region of the tRNA^{Gln}. Extent of contamination of cytosolic 18S rRNA is indicated.

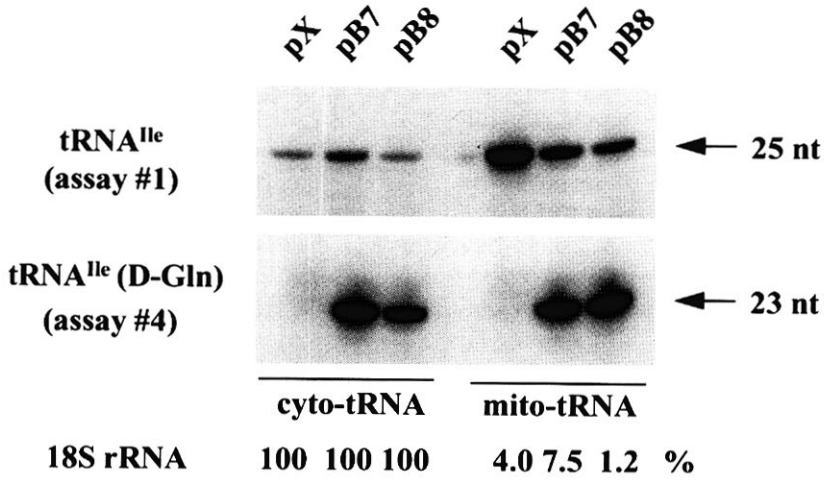

FIGURE 5. Expression and localization of the tRNA^{Ile}(D-Gln). Cyto-tRNA and mito-tRNA from the pX, pB7, and pB8 transformants were analyzed by using primer extension assays #1 and #4. Sizes of the expected extension products are indicated on the right. The pB7 construct contains the gene for tRNA^{Ile}(D-Gln) without the 5′-flanking region, and the pB8 construct contains the same gene with the 5′-flanking region of the tRNA^{Gln}. Extent of contamination of cytosolic 18S rRNA is indicated.

a 23-nt labeled product only with the tRNA^{Ile}(D-Gln) and not with the endogenous tRNA^{Ile} (Table 1). The presence of the 23-nt band in both cytosolic and mitochondrial lanes indicates that replacement of the D loop+stem did not eliminate the mitochondrial localization of this tRNA. As discussed for the pB5 and pB2 transformants, we speculate that the decrease of the labeled 25-nt product (assay #1, wt tRNA^{Ile}) in mito-tRNA fractions from the pB7 and pB8 transformants in comparison to the amount of this product in the pX transformant could be related to the presence of the plasmid-expressed tRNA in these cells and possible competition for importation.

The results also show that the presence (pB8) or absence (pB7) of a 5′-flanking sequence derived from the tRNA^{Gln} gene had no effect on the expression or subcellular localization of the mutated tRNAs, confirming the previous results.

Evidence for mitochondrial localization of the plasmid-expressed tRNA^{Gln}(D-Ile)

The purified mitochondrial fractions in Figure 6 were subjected to increasing concentrations of digitonin, which is known in other systems to selectively solubilize the outer mitochondrial membrane at low concentrations (Schnaitman & Greenawalt, 1968), and then digested with micrococcal nuclease prior to tRNA extraction and primer extension assay. The kinetics of digitonin-induced sensitivity to micrococcal nuclease digestion can be used to monitor the localization of specific tRNAs, because tRNAs attached to the outer membrane would be expected to be nuclease-sensitive at relatively low digitonin concentrations, whereas tRNAs within the inner matrix would not become nuclease-sensitive until higher digitonin concentrations. Due to the absence of appropriate outer membrane markers in the trypanosomatid system, the control consisted of 3′-end-labeling endogenous mito-tRNAs with [α-^{32}P]CTP

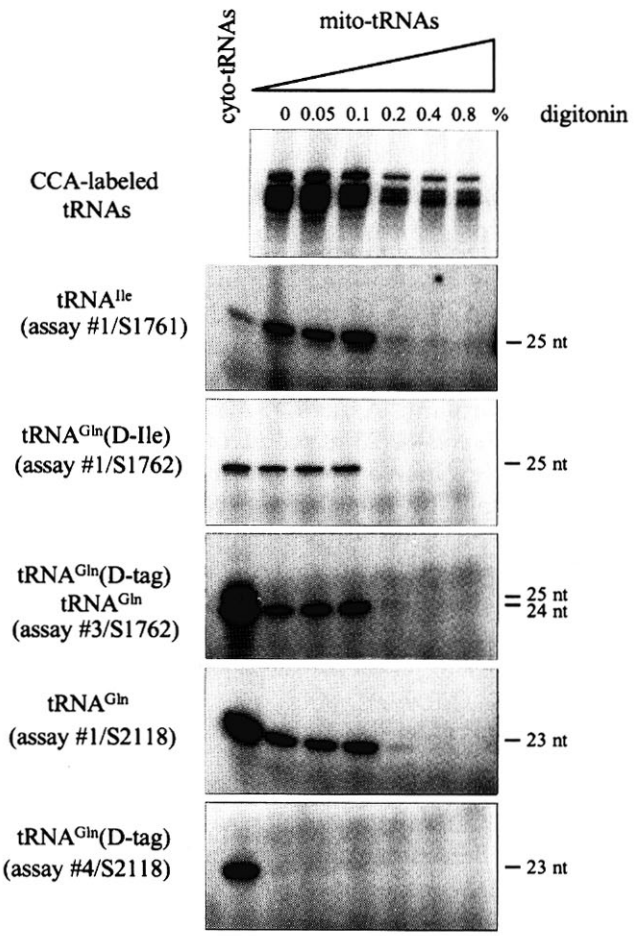

FIGURE 6. Evidence for intra-mitochondrial localization of tRNA^{Gln}(D-Ile). The isolated mitochondrial fractions from wt cells and from the pB4 and pB10 transformants expressing the indicated tRNAs were incubated with increasing concentrations of digitonin during the digestion with micrococcal nuclease and posterior extraction of tRNA. The indicated primer extension assays were then performed and the products analyzed in denaturing gel. In the upper panel, endogenous mito-tRNAs were 3′ end-labeled by incubation of the mitochondrial fraction with [α-^{32}P]CTP prior to digitonin/micrococcal nuclease treatment. In the remaining panels, the first lane contains cyto-tRNA and the remaining lanes mito-tRNA. Sizes of the expected extension products are indicated on the right.

(Hancock et al., 1992) in the intact organelles, and then following the concentration-dependence of digitonin-induced sensitivity to micrococcal nuclease digestion of the labeled matrix-localized tRNAs. As shown in Figure 6, the wt $tRNA^{Ile}$, measured with assay #1 (Table 1), showed the same kinetics of solubilization by digitonin as the CCA-labeled, matrix-localized tRNAs. This provides additional evidence that the wt $tRNA^{Ile}$ is within the mitochondrial matrix and is not attached in some way to the outside of the vesicles.

The isoacceptor $tRNA^{Gln}$(UUC) detected in mitochondrial tRNA (wt $tRNA^{Gln}$: assay #3, 24-nt product with primer S1762, and assay #1 with primer S2118) has the same kinetics of susceptibility to micrococcal nuclease in digitonin solutions as the wt $tRNA^{Ile}$ and the CCA-labeled, matrix-localized tRNAs.

Identical results were obtained with the $tRNA^{Gln}$(D-Ile), confirming that this mutated tRNA is indeed localized within the mitochondrial matrix. On the other hand, the $tRNA^{Gln}$(D-tag), assayed using either the S1762 primer or the S2118 primer, was not detectable in the mitochondrial fraction, confirming the exclusively cytosolic localization of the tagged $tRNA^{Gln}$.

To eliminate the possibility of the observed mitochondrial localization of the $tRNA^{Gln}$(D-Ile) being due to cytosolic contamination, transfection experiments were performed using the plasmid pB11, which contains the $tRNA^{Gln}$(D-tag) and the $tRNA^{Gln}$(D-Ile) genes in tandem. The results in Figure 7 show that the plasmid-encoded tRNA genes were co-expressed in the pB11 transformant. Assays #3 (25-nt product with primer S1762) and #4 (23-nt product with primer S2118) showed an exclusive cytosolic localization of the $tRNA^{Gln}$(D-tag) by the absence of these extention products in pB11 mito-tRNA samples, and assay #1 (25-nt product with primer S1762) showed a definite mitochondrial localization of the $tRNA^{Gln}$(D-Ile) by the presence of the extention product in the pB11 mito-tRNA sample.

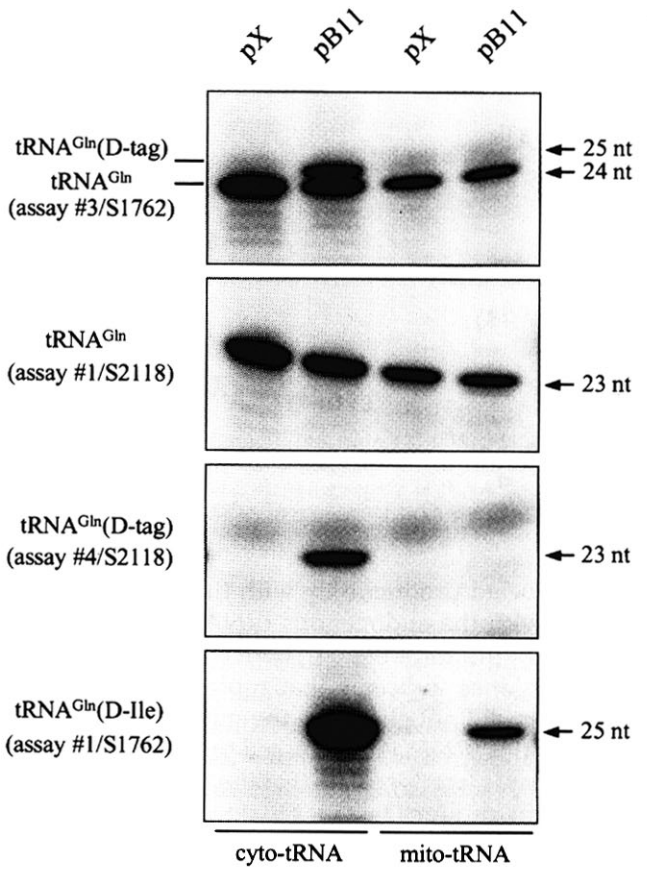

FIGURE 7. Co-expression of the $tRNA^{Gln}$(D-tag) and the $tRNA^{Gln}$(D-Ile) from the same plasmid, and intracellular localization. Cyto-tRNA and mito-tRNA from the pX and pB11 transformants were analyzed by using the indicated primer extension assays for the presence of endogenous $tRNA^{Gln}$ and the co-expressed $tRNA^{Gln}$(D-tag) and $tRNA^{Gln}$(D-Ile). Sizes of the expected extension products are indicated on the right. The pB11 construct contains the gene for $tRNA^{Gln}$(D-tag) and the gene for $tRNA^{Gln}$(D-Ile) in tandem and lacking the 5′-flanking region.

DISCUSSION

We have shown that *L. tarentolae* cells transfected with plasmids containing tagged tRNA genes express the tRNAs that become localized in the cytosolic and/or mitochondrial compartments, depending on specific tRNA sequences. The evidence for the localization of the tagged $tRNA^{Ile}$ within the mitochondrial compartment is the comigration of the tRNA with a isopycnically purified, DNase I-, and micrococcal nuclease-digested mitochondrial fraction (Braly et al., 1974) and the kinetics of digitonin-induced sensitivity to micrococcal nuclease digestion.

Hancock et al. (1992) and Hancock and Hajduk (1992) have suggested that 5′-extended tRNAs present in the mitochondrion of *T. brucei* represent precursor molecules that are then processed to mature tRNAs by an RNase P-like activity. This would imply that these 5′-extended tRNAs represent the substrate for the importation apparatus. Our results clearly demonstrate that the genomic 5′-flanking sequence of the tagged $tRNA^{Ile}$ gene is not necessary either for in vivo expression from a plasmid or for importation of the tRNA into the mitochondrion. Likewise, the genomic 5′-flanking sequence of the cytosolic-localized tagged $tRNA^{Gln}$ gene is not necessary for in vivo expression from plasmid DNA. This evidence suggests that the signal for importation is within the tRNA sequence itself and is consistent with the results of Hauser and Schneider (1995) in *T. brucei*.

The mutation analysis in this paper indicated that specific sequence determinants for importation are localized within the $tRNA^{Ile}$ D loop region, because swapping the $tRNA^{Ile}$ D loop+stem sequence produced a partial mitochondrial targeting of the $tRNA^{Gln}$. The amount of the $tRNA^{Gln}$(D-Ile) within

the mitochondrial fraction was equivalent to the amount of the endogenous $tRNA^{Ile}$ and also to the amount of the plasmid-expressed $tRNA^{Ile}$(D-tag). A substantial portion of the $tRNA^{Gln}$(D-Ile) was also localized in the cytosolic fraction. We speculate that this is due to overexpression of the tRNA from the multicopy plasmid and subsequent saturation of the mitochondrial import receptors. An observed decrease in the absolute amount of the endogenous $tRNA^{Ile}$ in the mitochondrial fraction of transformants and an increase in the amount of cytosolic-localized plasmid-expressed $tRNA^{Ile}$(D-tag) could also be due to this same phenomenon.

It is clear that the localization of the tagged $tRNA^{Gln}$ is entirely cytosolic and that the eight single-nucleotide changes within the D loop+stem (in comparison to the $tRNA^{Gln}$(D-tag) sequence versus the $tRNA^{Gln}$(D-Ile) sequence shown in Fig. 1A) produced a mitochondrial localization. A comparison of the sequence of the isoacceptor $tRNA^{Gln}$(UUG), which is normally partially mitochondrial-localized, with the sequence of wt $tRNA^{Gln}$(CUG), shows a single C to U substitution within the D loop in addition to several additional substitutions elsewhere in the molecule (Shi et al., 1994). It is of interest that this C to U substitution is one of the eight changes involved in producing a mitochondrial-localization of the tagged $tRNA^{Gln}$ (Table 1).

Specific D loop nucleotides have been shown to interact with other nucleotides, especially within the TψC loop, and these interactions affect the tertiary structure of the tRNA (Rich & RajBhandary, 1976). We speculate that the failure of the $tRNA^{Gln}$ D loop+stem sequence to inhibit mitochondrial importation of the $tRNA^{Ile}$ suggests that tertiary interactions of D loop nucleotides with other nucleotides in the molecule, or additional sequence elements, and not just the D loop sequence motif itself, may play a role in determining targeting to the mitochondrion. This is consistent with the previous report of Chen et al. (1994) that a four-nucleotide insertion in the $tRNA^{Thr}$(AGU) variable loop abolished mitochondrial targeting of this tRNA in transfected *L. tarentolae* cells.

In conclusion, we have established that signals for tRNA targeting to the mitochondrion in *L. tarentolae* reside within the tRNA itself, and that the D loop and stem region is important for the conversion of a nonmitochondrial-targeted tRNA into a mitochondrial-targeted tRNA. This opens the door for an in vivo analysis of the mechanism of the transport of tRNAs across the mitochondrial double membrane.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides for gene constructs, mutagenesis, PCR, primer extension, and hybridization were synthesized by standard phosphoramidite methods and purified by thin-layer chromatography. The following oligonucleotides were utilized in this study.

S662: GACTACAATGGTCTCTAATC (20mer); antisense of 18S rRNA;
S1446: GCTCCCGTGGTCTAGTTGGCACGGACGCTGGTC TTATGAACCGGATGTCGCGGGTTCGAGCCCC (64mer); sense strand of $tRNA^{Ile}$(D-tag);
S1447: TGCTCCCGGCGGGGCTCGAACCCGCGACATCC GGTTCATAAGACCAGCGTCCGTGCCAACTAGA (64mer); antisense strand of $tRNA^{Ile}$(D-tag);
S1475: GTAGGAGTGCTTTTCCGCC (19mer); bridge oligo for 3′-flanking Ile and antisense strand of $tRNA^{Ile}$;
S1476: TATAGGAGCGTGGTACATA (19mer); bridge oligo for 5′-flanking Ile and sense strand of $tRNA^{Ile}$(D-tag);
S1477: TATAGGAGCGCCTGATCAT (19mer); bridge oligo for 5′-flanking Gln and sense strand of $tRNA^{Ile}$(D-tag);
S1478: GATCCTCACACGAACTTGGGTCTCCAGGGCGTT CACAACTCCCCACCTACATCCATATTCAGTATGTAC CAC (72mer); 5′-flanking region of $tRNA^{Ile}$;
S1481: GATCCGTTATGGGCTAACCGGGGGGGGGGGG CTCCAGTTATACTGTCACCTTCATCATCCTTGTATGAT CAGGC (74mer); 5′-flanking region of $tRNA^{Gln}$;
S1494: CCGGGTTTTTTCCGTGTTGGGGATTTTGGGGCG GAAAAG (39mer); 3′-flanking region of $tRNA^{Ile}$;
S1502: CCACGGGAGCGTGGTACATA (20mer); bridge oligo for 5′-flanking Ile and sense strand of $tRNA^{Gln}$(D-tag);
S1503: CCACGGGAGCGCCTGATCAT (20mer); bridge oligo for 5′-flanking Gln and sense strand of $tRNA^{Gln}$(D-tag);
S1504: GCCGGGAGCACTTTTCCGCC (20mer); bridge oligo for 3′-flanking Ile and antisense strand of $tRNA^{Gln}$;
S1686: GCTCCTATAGTGTAGCGGAACTCACCTCGGACT CTGAATCCGATAACCCTGGTTCGAGTCCAG (63mer); sense strand of $tRNA^{Gln}$(D-tag);
S1687: CACTCCTACCTGGACTCGAACCAGGGTTATCGG ATTCAGAGTCCGAGGTGAGTTCCGCTACAC (63mer); antisense strand of $tRNA^{Gln}$(D-tag);
S1752: TAATACGACTCACTATAGCTCCCGTGGTCTAGTT GG (36mer); 5′ PCR primer of $tRNA^{Ile}$
S1761: TCCGGTTCATAAGACCAGCGT (21mer); antisense oligo of anticodon loop+stem of $tRNA^{Ile}$;
S1762: ATCGGATTCAGAGTCCGAGGT (21mer); antisense oligo of anticodon loop+stem of $tRNA^{Gln}$;
S1876: CGGGATCCTAATACGACTCACTATAGTTATGGGC TAACCGGGGGG (45mer); 5′ PCR primer of 5′-flanking region of $tRNA^{Gln}$;
S1877: TCCCCGGGTTTTTTCCGTGTTGGGG (25mer); 3′ PCR primer of 3′-flanking region of $tRNA^{Ile}$;
S1878: CGGGATCCTAATACGACTCACTATAGCTCCTATA GTGTAGCGG (43mer); 5′ PCR primer of $tRNA^{Gln}$;
S1886: GCGTGATAACCGCTACACCACGGGAGCGCCTG ATCATAC (39mer); antisense strand of $tRNA^{Ile}$ for mutagenesis of D loop;
S1887: TGGTGTAGCGGTTATCACGCTGGTCTTATGAAC CGG (36mer); sense strand of $tRNA^{Ile}$ for mutagenesis of D loop;
S1888: TAGTCTAGTTGGTTAGGACCTCGGACTCTGAAT CCGATAAC (41mer); sense strand of $tRNA^{Gln}$ for mutagenesis of D loop;
S1889: AGGTCCTAACCAACTAGACTATAGGAGCGCCTG ATCATAC (40mer); antisense strand of $tRNA^{Gln}$ for mutagenesis of D loop;

S1906: CGGGATCCTAATACGACTCACTATAGCTCCT ATAGTCTAGTTGGTT (46mer); 5′ PCR primer of $tRNA^{Ile}$(D-Gln);
S1907: CGGGATCCTAATACGACTCACTATAGCTCCC GTGGTGTAGCGGTTA (46 mer); 5′ PCR primer of $tRNA^{Gln}$(D-Ile);
S2118: CGGATTCAGAGTCCGAGGTGA (21 mer); antisense oligo of anticodon loop+stem of $tRNA^{Gln}$.

Gene constructions

The first step in the construction of the tagged tRNA genes was a phosphorylation of 30 pmol of each oligonucleotide for the structural genes (S1446, S1447, S1686, and S1687) using ATP and polynucleotide kinase (BRL). The phosphorylated oligonucleotides were ligated with the 5′-flanking region oligonucleotides or 3′-flanking region oligonucleotide in the presence of the corresponding bridge oligonucleotide, using T4 DNA ligase (BRL). The ligated oligonucleotides were gel-eluted and annealed in pairs, and filling-in and phosphorylation reactions were performed with Klenow DNA polymerase I and polynucleotide kinase (BRL), respectively.

PCR amplifications were performed in 50-μL reactions containing 50 mM KCl, 10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.1 mg/mL BSA, 200 μM of each dNTP, 0.2 μM of each primer, 2.5 U AmpliTaq (Perkin Elmer), and appropriate template DNA, using the GeneAmp System PCR9600 (Perkin Elmer). PCR conditions were 2 min at 94 °C, followed by 30 cycles at 94 °C for 20 s, 55 °C or 60 °C for 20 s, and 72 °C for 20 s.

To mutagenize the D loop+stem of the $tRNA^{Ile}$ and $tRNA^{Gln}$, PCR was performed using the 5′-flanking region of the $tRNA^{Gln}$ and the 3′-flanking region of the $tRNA^{Ile}$ primers, with the corresponding internal mutant primers. PCR products were treated with T4 DNA polymerase at 12 °C for 15 min to remove the 3′-A overhanging bases, and purified by gel electrophoresis. These products were mixed together in pairs and five cycles of PCR reaction without primers were performed to increase the number of molecules containing the full-length gene, followed by addition of primers and PCR amplification, and subsequent treatment with T4 DNA polymerase and polynucleotide kinase.

Genes without the 5′-flanking region were obtained by PCR using primers located in the 5′ region of the respective tRNA genes and a 3′-flanking region primer, followed by treatment with T4 DNA polymerase and polynucleotide kinase (BRL).

Plasmid constructions

All gene constructs were cloned into dephosphorylated *Sma*I-digested pUC18 plasmid (Pharmacia). After transformation of *E. coli* XL1 Blue cells (Stratagene), plasmid DNA was extracted by a minilysate boiling method and selection of recombinant plasmids was performed by restriction enzyme digestion and analysis in agarose gel (Sambrook et al., 1989). The tRNA genes were sequenced with the Sequenase version 2.0 kit (USB), using [α-^{35}S]dATP (NEN), following the manufacturer's instructions. The *Bam*H I/*Sal* I 3.2-kb fragment of the pX plasmid (LeBowitz et al., 1990) (kindly provided by Dr. Stephen Beverley) containing the DHFR/Neo^r gene was cloned into each of the tRNA plasmids to obtain the final constructions.

Transfection of *L. tarentolae* cells

L. tarentolae (UC strain) cells were grown as described (Simpson & Braly, 1970) to late log phase (1–2 × 10^8 cells/mL). Transfection was performed by electroporation as described (Coburn et al., 1991), using 2 × 10^7 cells per electroporation with 5–10 μg DNA, and applying single pulses of 500 μF and 2.25 kV/cm in a BTX Electroporator. Electroporated cells were transferred to a 50-mL bottle culture containing 10-mL of brain heart infusion medium (Difco) supplemented with 10 μg/mL hemin, and incubated at 27 °C for 24 h. Geneticin G418 (BRL) was added to a final concentration of 50 μg/mL. The cultures were diluted 20-fold every 2–3 days for a total of 5 transfers for DNA isolation and 10 transfers for RNA isolation.

RNA isolation

L. tarentolae transformants were grown in cultures containing 40–50 μg/mL G418 to late log phase (1–2 × 10^8 cells/mL). The isolation of the kinetoplast-mitochondrial fraction was performed by isopycnic flotation in Renografin density gradients, as described (Braly et al., 1974). The mitochondria were obtained from cells ruptured under hypotonic conditions and rapidly restored to isotonic conditions and it is possible that the outer membrane was fragmented, but there is good evidence that the inner membrane was intact. During the isolation procedure, the mitochondria were treated with DNase I to remove any nuclear DNA and this treatment did not affect the endogenous kinetoplast DNA. The mitochondrial fraction was also treated with 600 units micrococcal nuclease for 1 h at 4 °C to remove any contaminating cytoplasmic RNA. This treatment has no effect on the endogenous mitochondrial mRNAs, rRNAs, or guide RNAs. Cytoplasmic RNA was isolated from the supernatant obtained after hypotonic lysis and centrifugation, and mitochondrial (kinetoplast) RNA was isolated from the purified mitochondrial fraction (Simpson & Simpson, 1978). It should be noted that the cytosolic RNA fraction could be contaminated with nuclear RNA from nuclei disrupted by the hypotonic lysis. The tRNA fractions (Simpson et al., 1989) were gel-isolated and utilized in the primer extension experiments.

Primer extension assays

The basic strategy for primer extension experiments was to anneal 10 pmol of a specific primer for $tRNA^{Ile}$ (oligonucleotide S1761) or for $tRNA^{Gln}$ (oligonucleotide S1762 or S2118), complementary to the anticodon loop+stem, to 100 ng of the tRNA fraction in reverse transcription buffer (50 mM Tris, pH 8.6, 60 mM NaCl, 10 mM DTT), in a final volume of 5 μL. The samples were heated to 90 °C for 1 min and transferred to room temperature. One microliter of 30 mM $MgCl_2$ was added and 2 μL was transferred to a tube containing 5 μCi of [α^{32}P]dNTP, 375 μM dNTP, 200 μM ddNTP, and 1 U AMV-RT

(Promega), and the reactions were incubated at 44 °C for 30 min. The extension products were analyzed in 12% polyacrylamide–8 M urea gels and subsequent autoradiography. 5′-^{32}P-labeled *MspI*-digested pBR322 DNA was utilized as a molecular weight marker.

Assay for contamination of the mitochondrial fraction with cytosolic material

Northern blot analysis was performed by electroblotting the gel containing the total cyto-RNA and mito-RNA in a Hoefer Model SE400 apparatus onto nylon membranes (Nytran Plus 0.2 mm, Schleicher & Schuell; or MagnaGraph 0.45 mm, Micron Separations Inc.) for 24 h at 0.5 mA in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). The RNA was crosslinked to the membrane using a UV Stratalinker system (Stratagene). Prehybridizations and hybridizations were performed at 45 °C in 6× SSC, 5× Denhardt's, 0.4% SDS, 0.05% tetrasodium pyrophosphate, and 100 μg/mL sonicated and denatured salmon sperm DNA. Blots were probed with oligonucleotide S662, for detection of 18S rRNA, which was 5′ end-labeled using [γ^{32}P]ATP (NEN) and polynucleotide kinase (BRL). Blots were washed once for 10 min at room temperature in 6× SSC and 0.1% SDS solution, once for 10 min at room temperature, and twice for 30 min at 50 °C in tetramethylammonium chloride solution (3 M tetramethylammonium chloride, 2.0 mM EDTA, 50 mM Tris HCl, pH 8.0, 1.0% SDS) (Wood et al., 1985); quantification was performed on Molecular Dynamics PhosphoImager.

Digitonin experiments

A kinetoplast-mitochondrial fraction was isolated from 4 L late log phase cultures of wt, pB4, and pB10 transfected *L. tarentolae* cells by isopycnic flotation in Renografin density gradients (Braly et al., 1974). Micrococcal nuclease (600 units) and 5 mM $CaCl_2$ were added to the mitochondrial fraction in 2.5 mL of 0.25 M sucrose, 20 mM Tris HCl, pH 7.9, 2 mM $MgCl_2$. The mitochondrial fraction was divided into six aliquots and digitonin was added to five of these at final concentrations of 0.05, 0.1, 0.2, 0.4, and 0.8%. The solutions were incubated 20 min at 27 °C and then the mitochondria were washed twice with 0.25 mM sucrose, 20 mM Tris HCl, pH 7.9, 2 mM EDTA. Total mito-RNA was isolated and the tRNA fraction gel-isolated. Primer extension analysis was performed as described above.

Metabolic 3′ CCA end-labeling of endogenous tRNA within mitochondrial vesicles with [α-^{32}P]CTP was performed as described (Hancock et al., 1992). The mitochondrial fraction was then washed with 0.25 mM sucrose, 20 mM Tris HCl, pH 7.9, 2 mM EDTA. Total mito-RNA was isolated by phenol/chloroform extraction and ethanol precipitation. The RNA was analyzed by gel electrophoresis and autoradiography.

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