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Differential localization of nuclear-encoded tRNAs between the cytosol and mitochondrion in *Leishmania tarentolae*

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ABSTRACT

All mitochondrial tRNAs of the kinetoplastid protozoan *Leishmania tarentolae* are encoded in the nucleus and are imported from the cytosol into the mitochondrion. We previously reported the partitioning of five tRNAs and found that all were shared between the two compartments to different extents. To increase our knowledge of the tRNAs of this organism, and to attempt to understand the signals involved in their subcellular localization, a method to RT-PCR amplify new tRNAs was developed. Various tRNAs were 3′ polyadenylated and reverse transcribed with a sequence-tagged primer. The cDNA was tagged by ligation to an anchor oligonucleotide, and the resulting double-tagged cDNA was amplified by PCR. Four new tRNAs were obtained, bringing to 20 the total number of *L. tarentolae* tRNAs identified to date. The subcellular localization of 17 tRNAs was quantitatively analyzed by two-dimensional gel electrophoresis and northern hybridization. In general, the previously suggested operational classification of tRNAs into three groups (mainly cytosolic, mainly mitochondrial, and shared between the two compartments) is still valid, but the relative abundance of each tRNA in the cytosol or mitochondrion varied greatly as did the level of expression.

Keywords: *Leishmania tarentolae*; oligonucleotide ligation; RNA quantitation; subcellular localization; tRNA importation

INTRODUCTION

The importation of nuclear-encoded tRNAs into mitochondria has been described in a wide variety of organisms including yeast (Kazakova et al., 1999), ciliates (Rusconi & Cech, 1996a), plants (Dietrich et al., 1992), animals (Beagley et al., 1998), and trypanosomatids (Simpson et al., 1989; Hancock & Hajduk, 1990; Schneider et al., 1994; Lima & Simpson, 1996; see Schneider & Maréchal-Drouard, 2000, for a recent review). The extent of this process ranges from the importation of one tRNA not required for mitochondrial translation (Tarassov & Martin, 1996) to the importation of tRNAs absent from the mitochondrial genome and therefore required for translation.

Elucidation of the common features of imported tRNAs is important to understanding the specificity of this process in various organisms. In *Tetrahymena*, a single nucleotide change in the anticodon (UUA to UUG) of a nonimported $tRNA^{Gln}$ is sufficient to confer importation (Rusconi & Cech, 1996b). Similarly, in *Saccharomyces*, the CUU anticodon as well as a G:C base pair in the acceptor stem are major determinants of the importation of one of the two lysyl tRNAs encoded in the nuclear genome (Kazakova et al., 1999).

The mitochondrial genomes of the trypanosomatids, *Leishmania tarentolae* and *Trypanosoma brucei*, contain no tRNA genes (Simpson et al., 1989; Hancock & Hajduk, 1990), making it necessary to import all of the mitochondrial tRNAs from the cytosol after their transcription from nuclear-encoded genes. However, the process shows some selectivity, as some tRNAs are imported into the mitochondrion more efficiently than others (Rubio et al., 2000). It has been suggested that the D-arm of tRNAs in *Leishmania* acts as a signal influencing tRNA import (Mahapatra et al., 1998). This is supported by in vivo and in vitro experiments in which the swapping of the D-arm sequences between the imported $tRNA^{Ile}$ and the cytosolic $tRNA^{Gln}$ reverses

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Abbreviations: RT: reverse transcription; RACE: rapid amplification of cDNA ends.

their respective import phenotypes (Lima & Simpson, 1996; Rubio et al., 2000). However, the fact that both $tRNA^{Ile}$ and $tRNA^{Gln}$ D-arm minihelices are imported in vitro demonstrates the importance of tertiary tRNA structure rather than specific D-arm sequences (Rubio et al., 2000). Additionally, an analysis of various *Leishmania* tRNAs showed no correlation between import phenotype and any conserved D-loop sequence elements (Suyama et al., 1998).

In *T. brucei*, RT-PCR experiments have suggested that the substrate for mitochondrial importation in one instance, and perhaps in general, is a dimeric tRNA precursor transcript (LeBlanc et al., 1999; Yermovsky-Kammerer & Hajduk, 1999). However, studies in *T. brucei* have shown that heterologous tRNAs can be imported into the mitochondrion in vivo regardless of their genetic origin or genomic context (Hauser & Schneider, 1995). Furthermore, precursor tRNA transcripts do not seem to be substrates for tRNA import in *L. tarentolae* either in vivo or in vitro, as both 5′- and 3′-end processing of several mitochondrial tRNAs was shown to occur in the nucleus (Kapushoc et al., 2000).

Identification of mitochondrial tRNA import or anti-import determinants in trypanosomatids is difficult, as the sequences of only a limited number of the tRNAs from these organisms are known. Methods to determine new tRNA sequences directly via base-specific cleavage provide limited information, and direct sequencing of RNA by dideoxy chain termination requires knowledge of at least some of the sequence of the RNA in question for primer design. Although there are several methods to clone unknown cDNA ends by 5′ and 3′ rapid amplification of cDNA ends (RACE; Frohman et al., 1988; Schaefer, 1995), these methods also require some knowledge of the RNA sequence for primer design. A recently developed RNA sequencing technique that makes use of ϕ6 RNA polymerase has the potential to directly and completely sequence unknown RNAs, but requires homogenous RNA templates with uniform 3′ ends (Makeyev & Bamford, 2001).

To obtain additional tRNA sequences from *L. tarentolae*, we have investigated several methods to clone novel tRNAs that may prove of general utility. Furthermore, to more accurately define the import phenotypes of the tRNAs of *L. tarentolae*, we have quantitatively measured the subcellular localization of each tRNA currently known in this organism.

RESULTS

Cloning new tRNAs from total mitochondrial tRNA using a 3′- and 5′-RACE method

The method to clone new tRNAs, as described in Figure 1, entails addition of a primer-binding site to the 3′ end of gel-purified tRNA by polyadenylation, followed by reverse transcription using a sequence tagged oligonucleotide complementary to the 3′ poly(A) tail and the 3′-end CCA of the tRNA. An anchor oligonucleotide is then ligated to the 3′ end of the cDNA, and the resulting double-tagged cDNA is then amplified by PCR.

The application of this RT-PCR amplification method to the cloning of gel-isolated mitochondrial tRNA from *L. tarentolae* is shown in Figure 2. Reverse transcription of the poly(A)$^+$ tRNA yielded cDNAs varying in length to approximately 110 nucleotides (Fig. 2A, lane 3). The heterogeneity in length is probably caused by premature termination of reverse transcription due to secondary structure and modified nucleotides of the tRNAs. The cDNA from 70 to 110 nt was used for the further cloning steps; the bands smaller than 70 nt include unextended primer (the lowest band) and primer extended by approximately 15 nt. The first PCR amplification of the double-tagged cDNAs yielded a mixture of PCR products (Fig. 2B, lane 2). To obtain more full-length tRNA sequences, the amplified cDNA greater than 90 bp was subjected to another PCR amplification (Fig. 2B, lane 3). The products of the second PCR that were greater than 100 bp were cloned and sequenced.

Sequencing 52 independent clones yielded 19 unique sequences. Of the 19 unique sequences, 9 were sequences of tRNAs already cloned from *L. tarentolae*, and 10 were sequences of new tRNAs (Fig. 3). All of these sequences contained the primary sequence and secondary structure elements conserved in tRNA molecules (Sprinzl et al., 1998), and can be identified as specific tRNAs by BLAST alignments with sequences of tRNAs from other organisms. As expected from the lengths of the PCR products, most of the sequences (9 of the 10 new sequences) represented tRNA 3′ ends truncated near the variable loop.

In addition to the nine partial tRNA sequences, a full-length sequence for the $tRNA^{Glu\text{-}1}$(TTC) was obtained, shown in Figure 4A. The sequence of this new tRNA was used to clone a 933-bp *Bam*HI-*Hin*dIII genomic fragment that contained two tRNA genes and a 5S rRNA gene (Fig. 4B). Part of this fragment (from 111 to 372, containing the $tRNA^{Val\text{-}1}$(CCA) and 5S rRNA genes) is identical to part of a previously cloned tRNA cluster from *L. tarentolae* (Suyama et al., 1998). Inspection of the $tRNA^{Glu\text{-}1}$ gene sequence revealed no differences as compared to the sequence of the RT-PCR product.

Cloning of a new tRNA from a two-dimensional gel spot

The RT-PCR cloning procedure was also performed using RNA isolated from a single two-dimensional gel spot (labeled “D” in Fig. 8). When the cDNA was ligated to the anchor oligonucleotide and PCR-amplified, a sin-

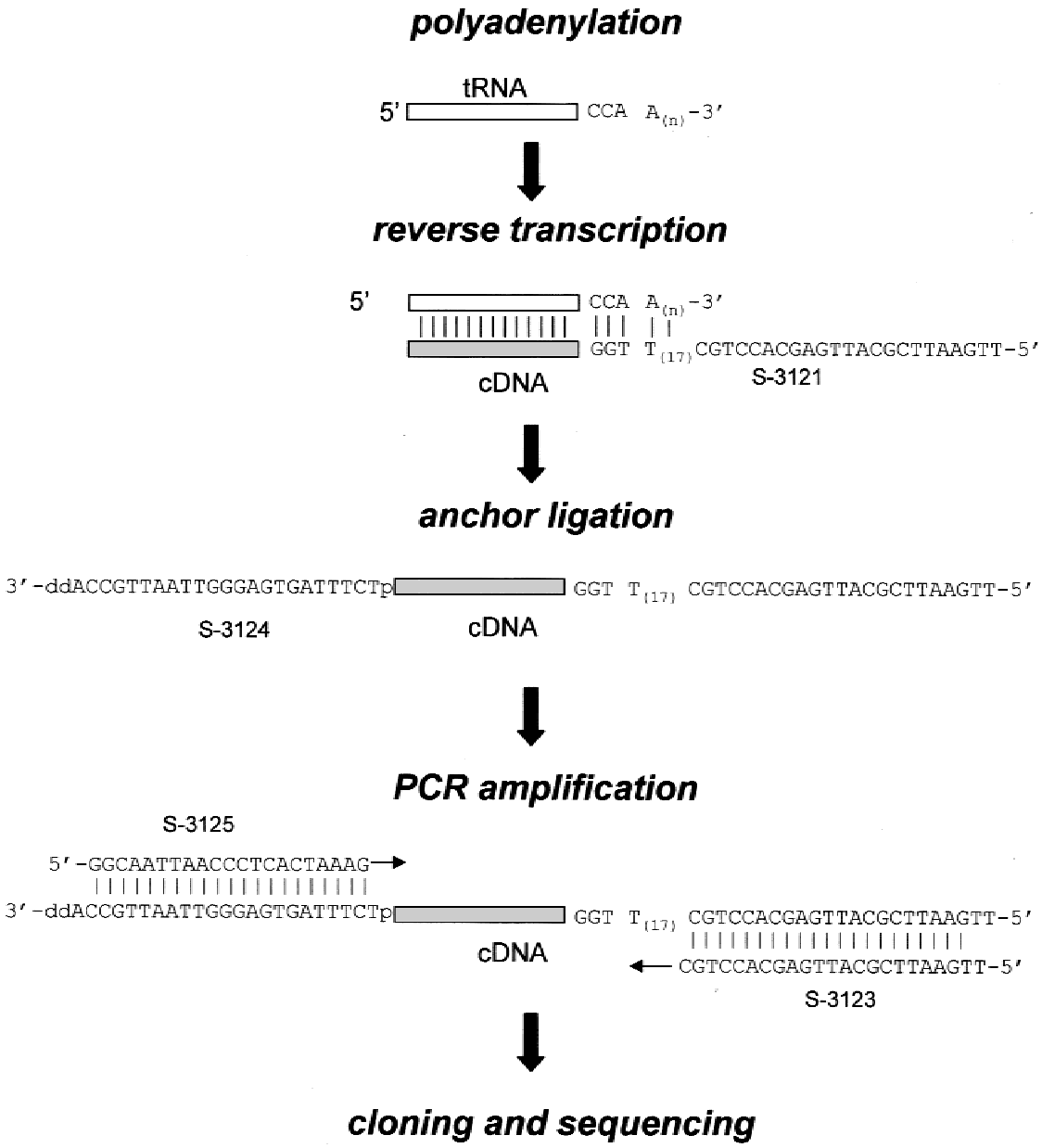

FIGURE 1. A scheme to RT-PCR amplify new tRNAs of unknown sequence. The tRNA (indicated by the open box) is polyadenylated with a mixture of yeast and *E. coli* poly(A) polymerases, and then reverse transcribed with oligonucleotide S-3121 (which hybridizes to the 3′ end CCA and poly(A) tail of the tRNA). The cDNA (indicated by the shaded box) is ligated to the anchor oligonucleotide S-3124, and the resulting double-tagged cDNA is PCR amplified with oligonucleotides S-3125 (which hybridizes to the anchor oligonucleotide sequence) and S-3123 (which matches the sequence tag contained in the oligonucleotide used for RT). Finally, PCR products are cloned and sequenced.

gle sequence was obtained (Fig. 5A). This sequence apparently represents the 3′ portion of a tRNA truncated in the anticodon loop and was used to design a reverse primer (S-3977) covering the variable loop region for reverse transcription and PCR amplification from total cell RNA (Fig. 5B). Two different full-length tRNA sequences were obtained from the PCR amplification of cDNA created by reverse transcription using oligonucleotide S-3977. The first sequence was the complete $tRNA^{Asp}$(GTC) (Fig. 5C); the second sequence could be identified as the $tRNA^{Glu\text{-}2}$ (CTC) and appeared to be the result of incorrect priming by the oligonucleotide during reverse transcription. The entire $tRNA^{Glu\text{-}2}$ (CTC) sequence was obtained from genomic sequence by constructing a hybridization probe (oligonucleotide S-4001) specific for the anticodon arm of the misprimed tRNA. This probe was used to clone a 1-kb genomic fragment from *L. tarentolae* that con-

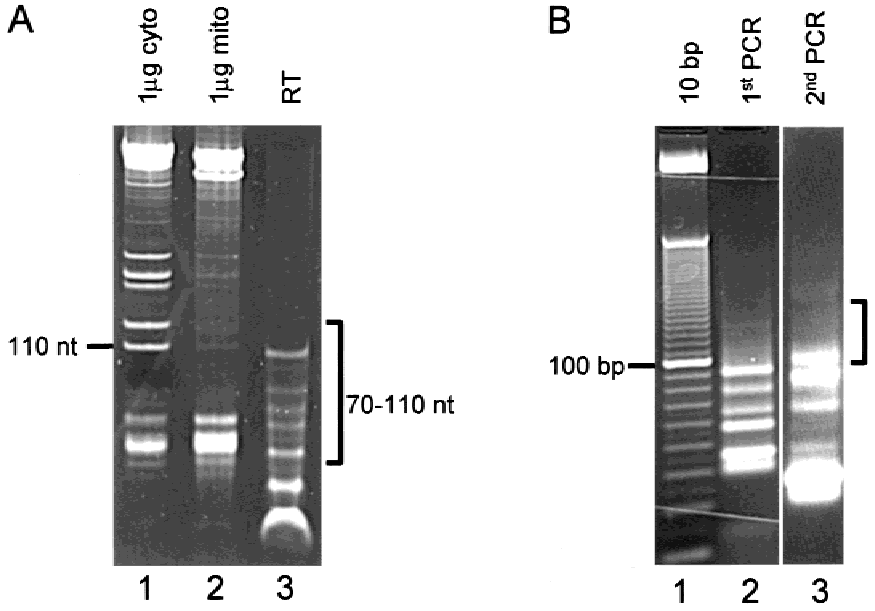

FIGURE 2. RT-PCR amplification of new tRNA sequences from *L. tarentolae*. **A**: *L. tarentolae* tRNAs are polyadenylated and reverse transcribed. Lanes 1 and 2: 1 μg of cytosolic RNA and 1 μg of mitochondrial RNA, respectively, for size markers; lane 3: a reverse transcription reaction using poly(A)$^+$ mitochondrial tRNA and oligonucleotide S-3121. The bracket indicates the cDNA purified for subsequent ligation to the anchor-oligo. **B**: Double-tagged cDNA is amplified by PCR. Lane 1: 10-bp ladder; lane 2: the double-tagged cDNA was PCR amplified using oligos S-3125 and S-3123; lane 3: a second PCR using oligos S-3125 and S-3123 and the first PCR product as a template. The bracket indicates the DNA that was gel purified, cloned, and sequenced.

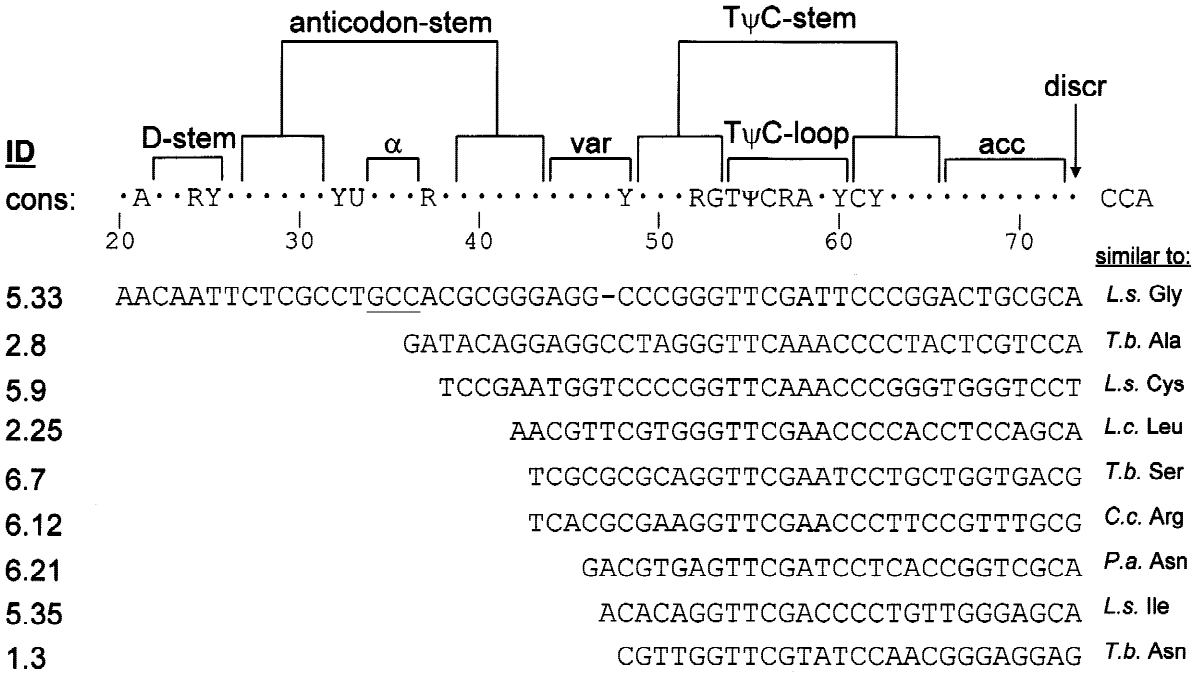

FIGURE 3. Several partial tRNA sequences obtained by RT-PCR. The top (labeled cons) shows the conserved primary sequence and secondary structure elements of a tRNA (α indicates the anticodon, var indicates the variable loop, acc indicates the acceptor stem, and discr indicates the discriminator nucleotide). The numbering is according to conventional tRNA nucleotide numbering (Sprinzl et al., 1998). In the first sequence (labeled 5.33′), – shows that the variable loop of this sequence is only 4 nt long; the underlined nucleotides point out the anticodon triplet. To the right of each sequence is identification of the most similar sequence identified (under "similar to") by BLAST searching (the standard three-letter amino acid code is used; *L.s.*: *Leptomonas seymouri*, *T.b.*: *Trypanosoma brucei*, *L.c.*: *Leptomonas collosoma*, *C.c.*: *Chondrus crispus*, *P.a.*: *Pichia angusta*).

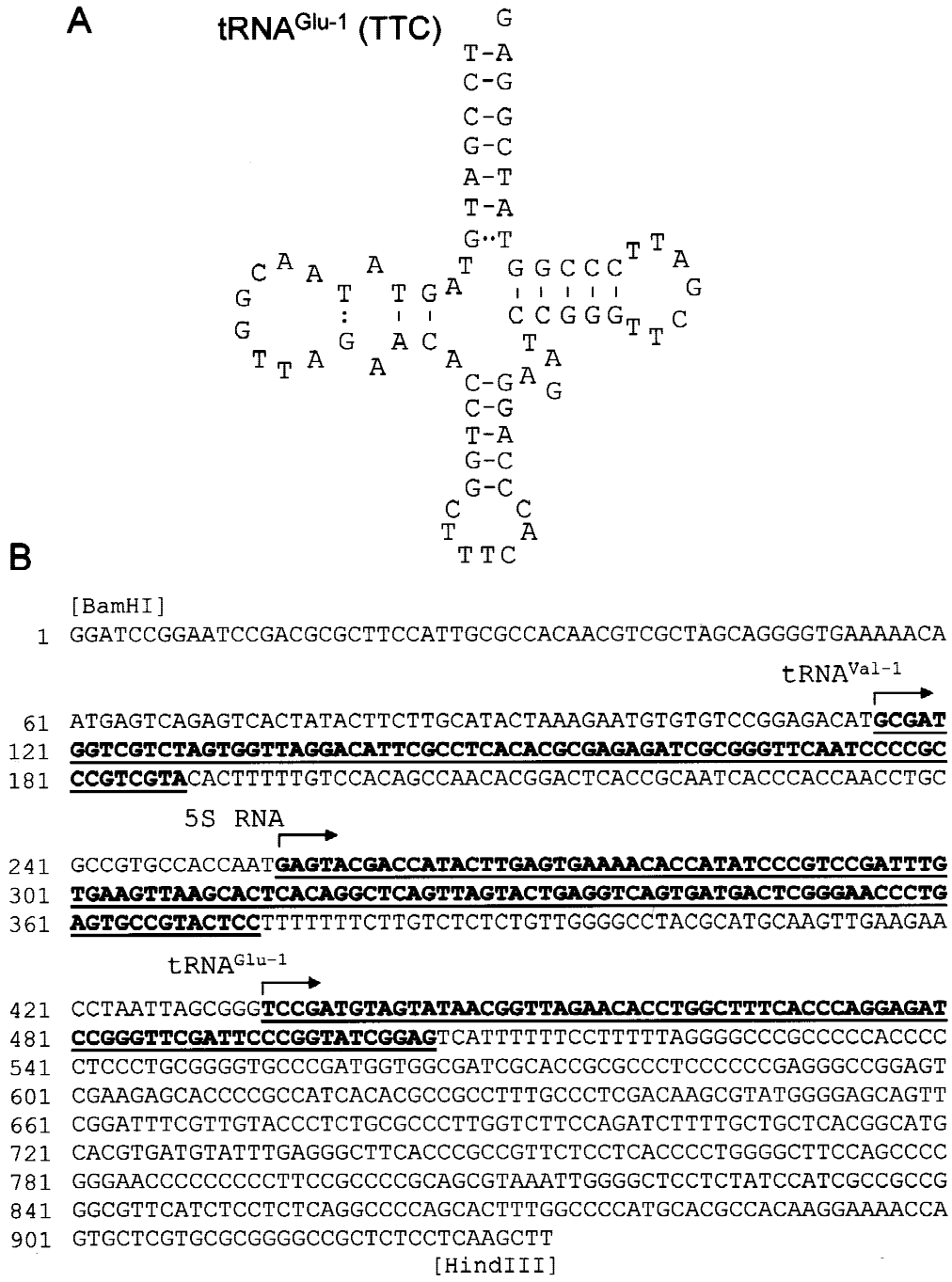

FIGURE 4. The tRNA^{Glu-1}(TTC) cloned in this study. **A**: The predicted clover-leaf structure of the tRNA^{Glu-1} that was cloned by the RT-PCR scheme described above. **B**: The nucleotide sequence of an *L. tarentolae* genomic clone encoding genes for two tRNAs and a 5S RNA. The RNA-coding sequences are shown in bold, underlined text. *Bam*HI and *Hin*dIII restriction sites are shown.

tained the entire tRNA^{Glu-2}(CTC) gene (Fig. 6). The similarity of the tRNA^{Glu-2}(CTC) and tRNA^{Asp}(GTC) sequences, especially in the TψC arm, may explain the observed mispriming with the S-3977 primer.

PCR amplification of the tRNA^{Asp}(GTC) gene from genomic DNA using oligonucleotides S-3977 and S-4012 yielded a gene sequence identical to that of the RT-PCR product in Figure 5B.

PCR amplification of new tRNA genes utilizing conservation of tRNA sequence motifs

We have also attempted to clone new tRNA genes by exploiting similarities in tRNA sequences and genomic organization of tRNA genes in related organisms. Based on the tail-to-tail organization of the tRNA^{Gly} and tRNA^{Pro}

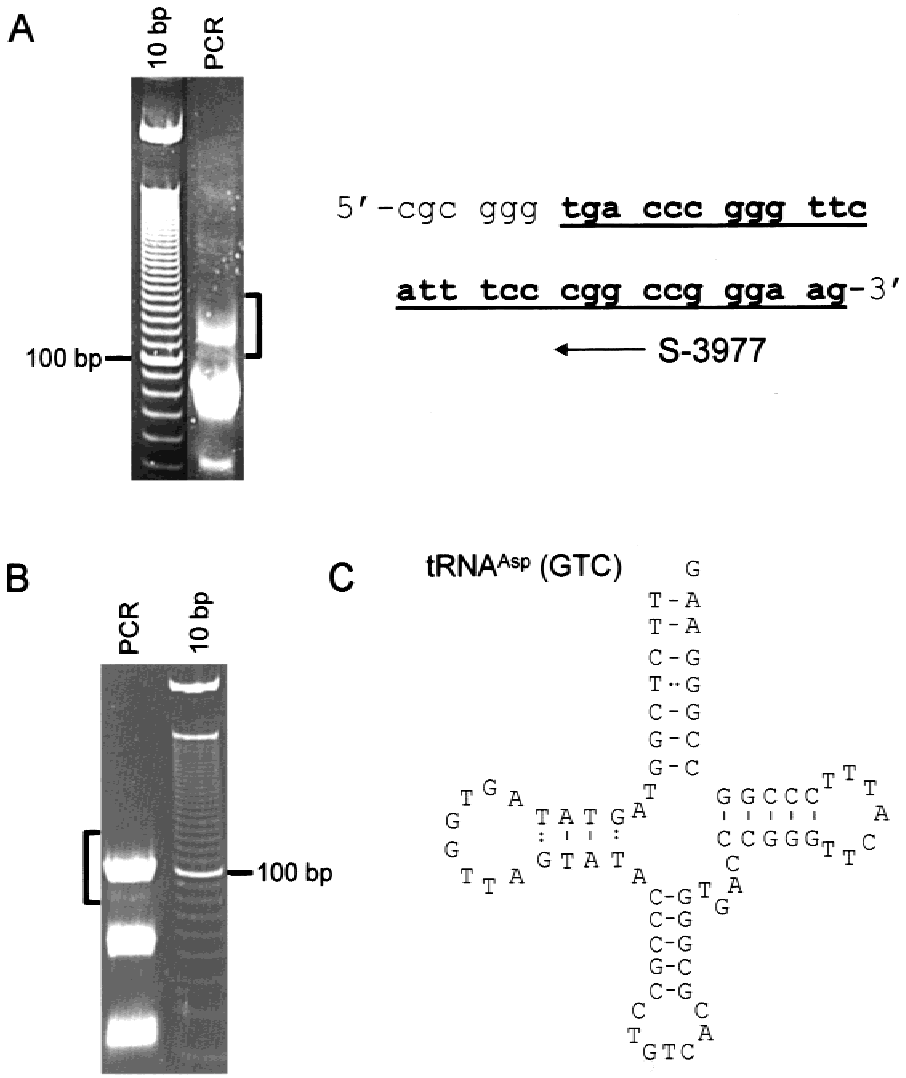

FIGURE 5. RT-PCR amplification of a single spot from a two-dimensional gel. **A**: A single spot was eluted from a two-dimensional gel, polyadenylated, and reverse transcribed with oligonucleotide S-3121. The resulting cDNA was ligated to the anchor oligonucleotide S-3124 and used as a template for PCR amplification with oligonucleotides S-3125 and S-3123. The bracket indicates the PCR product that was cloned and sequenced. The sequence of the PCR product (a putative tRNA 3′ end) without primer sequences is shown on the right; the bold, underlined portion indicates the sequence used to design S-3977 (a reverse oligonucleotide for the RT of a specific tRNA). **B**: The cDNA produced by reverse transcription of total RNA using oligonucleotide S-3977 was ligated to the anchor oligonucleotide and PCR amplified; the bracket indicates the PCR product that was cloned and sequenced. **C**: The predicted clover-leaf structure of the full-length $tRNA^{Asp}$(GTC) cloned by using oligonucleotide S-3977.

genes of *Leptomonas collosoma* and *Leptomonas seymouri* (GenBank AF204671 and AJ245951, respectively; Fig. 7A), and the fact that the tRNA sequences are similar to those from *Leishmania*, oligonucleotides S-3883 and S-3885 were constructed to PCR amplify a potential tRNA gene cluster from *L. tarentolae* genomic DNA. Two PCR products were obtained, one 200 bp and one 350 bp (Fig. 7A). Interestingly, the 200-bp fragment, which is the expected product size from the *Leptomonas* sequences, did not contain any tRNA genes, but the 350-bp product contained portions of two tRNAs as well as a complete $tRNA^{Thr\text{-}3}$(CGT) sequence (Fig. 7B,C). The partial *L. tarentolae* tRNA sequences in this PCR product appear to be from $tRNA^{Pro}$(CGG) and $tRNA^{Tyr}$(GTA) genes (Fig. 7B).

Quantitative analysis of tRNA distribution

The subcellular distribution of the new tRNAs identified in this study was examined by northern hybridization of two-dimensional gels, as shown in Figure 8. The oligonucleotide probes used were specific for each tRNA, yielding single spots in both the cytosolic and mitochondrial fractions for the $tRNA^{Glu\text{-}1}$(TTC), the $tRNA^{Glu\text{-}2}$(CTC), and the $tRNA^{Asp}$(GTC).

No hybridization signal was detected for the $tRNA^{Thr\text{-}3}$(CGT) or for the other two previously described threonyl tRNAs (S-3443, S-3447, and S-4003 were used as probes) in either fraction (not shown). It is unclear why we have not detected hybridization with any of the $tRNA^{Thr}$ probes. We did, however, amplify $tRNA^{Thr\text{-}1}$(AGT) and $tRNA^{Thr\text{-}2}$(TGT) in the RT-PCR scheme developed to find new tRNAs, and $tRNA^{Thr\text{-}3}$(CGT) was also detected by RT-PCR (Fig. 7D), indicating that all three threonyl tRNAs are expressed. Lack of hybridization of a *Leishmania* $tRNA^{Thr\text{-}2}$(TGT) probe has been described previously (Shi et al., 1994). Also, hybridization of a probe used to detect $tRNA^{Thr\text{-}1}$(AGT) showed that this tRNA migrates considerably faster than other tRNAs (Lye et al., 1993),

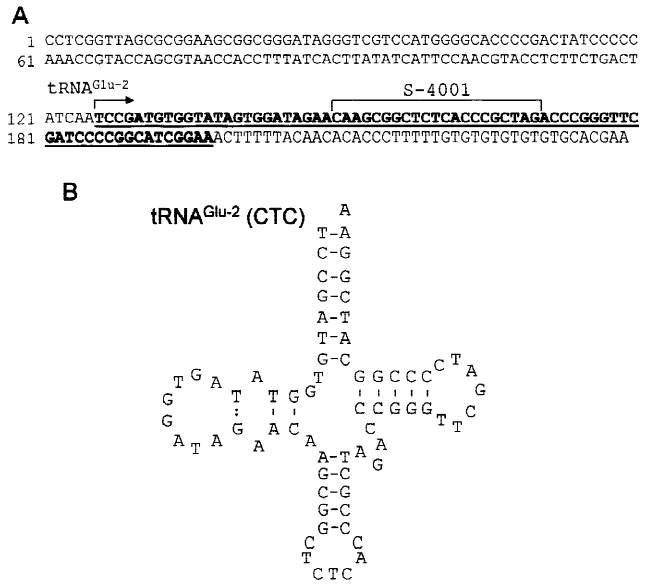

FIGURE 6. The tRNA^{Glu-2}(CTC) tRNA cloned in this study. **A**: The nucleotide sequence of a portion of an *L. tarentolae* genomic fragment containing the tRNA^{Glu-2} gene. The RNA-coding sequence is shown in bold, underlined text. The sequence detected by the oligonucleotide used to clone the fragment (oligonucleotide S-4001) is indicated. **B**: The predicted clover-leaf structure of the tRNA^{Glu-2}(CTC).

and it is therefore possible that this threonyl tRNA was not included in the second dimension gel.

Northern hybridizations of two-dimensional gels were also performed with oligonucleotide probes specific for all 16 previously described tRNAs (Lye et al., 1993), and these yielded single spots in both fractions (not shown). To determine the subcellular localization of these tRNAs, the hybridization signals were quantitated after exposure to a phosphorimager screen, yielding the localization ratios plotted in Figure 9. The relative subcellular steady-state distribution of each tRNA varies from three tRNAs that are enriched in the cytosol, to nine tRNAs that are approximately equally shared between the compartments, to five tRNAs that are somewhat enriched in the mitochondrion.

DISCUSSION

We have explored several methods to obtain new tRNAs from *L. tarentolae*. The RT-PCR-based method avoids the problems inherent in direct RNA sequencing caused by secondary structure and modified bases (Silberklang et al., 1979) and should prove useful for cloning novel RNAs in general. Also, addition of a primer-binding site to the cDNA via ligation of an anchor oligonucleotide, as opposed to homopolymeric tailing (Schmidt & Mueller, 1996), allows for more stringent conditions during the subsequent PCR step. The technique is readily adaptable for use with less structured RNAs that contain fewer modified nucleotides than tRNAs.

We used this method to clone three new tRNAs from total mitochondrial tRNA and also from a single tRNA spot from a two-dimensional gel. However, most of the sequences obtained by this method represented only the 3′ ends of the tRNAs, due to premature termination of reverse transcription usually around the variable loop. An additional RT-PCR with a primer covering the variable loop is then required to obtain the full-length sequence, or the 3′ sequence can be used as a probe to obtain a genomic fragment containing the tRNA gene. Nevertheless, the 3′ sequences are usually sufficient to recognize the tRNA by homology with database sequences. It is worth noting that the 3′ ends of tRNAs typically do not contain nucleotide modifications that might alter the sequence or tRNA identity of the resulting RT-PCR product (Auffinger & Westhof, 1998). Furthermore, we did not see any discrepancies between the sequences of RT-PCR products resulting from tRNAs already cloned from *L. tarentolae* and their corresponding genes, providing no evidence for the editing of tRNA 3′ ends as has been described in other organisms (Price & Gray, 1998; Yokobori & Pääbo, 1995).

The attempt to use the conserved tRNA sequence motifs from *Leptomonas* to clone a genomic fragment from *L. tarentolae* yielded an unexpected tRNA cluster containing a new tRNA^{Thr-3}(CGT) gene. This serendipitous amplification of a tRNA gene cluster illustrates the tendency towards mispriming when using primers based on the 3′ regions of tRNAs, due to sequence similarities in this region.

It is likely that bioinformatic analyses of the ongoing trypanosomatid genome sequencing projects should provide the remaining tRNAs in the near future, but experimental analysis will still be required to identify the subcellular localization of each tRNA.

The quantitative analysis of the intracellular distribution of 17 tRNAs presented in this study extends and improves previous analyses in several ways. The specific hybridization probes used in this study were generated against the anticodon arm portion of the tRNAs, which represents the most unique region of the tRNA. Previous studies have utilized probes that were generated against the TψC arm, which has a fairly conserved sequence among different tRNAs. We also employed two-dimensional gel separations of tRNAs, which allowed the identification of specific tRNAs from single spots. It should be noted that the ratios were not corrected for the absolute amount of tRNA present in the cytosol and the mitochondrion and therefore represent operational values obtained from analyzing equal amounts of each RNA fraction. Also, no assessment of the expression levels of different tRNAs was made in this study. For example, both tRNA^{Gln-1}(TTG) and tRNA^{Tyr}(GTA) have localization ratios of approximately 2.5 (Fig. 9), but the ethidium bromide-stained gel shows that the tRNA^{Gln-1}(TTG) is much more abundant (Fig. 8).

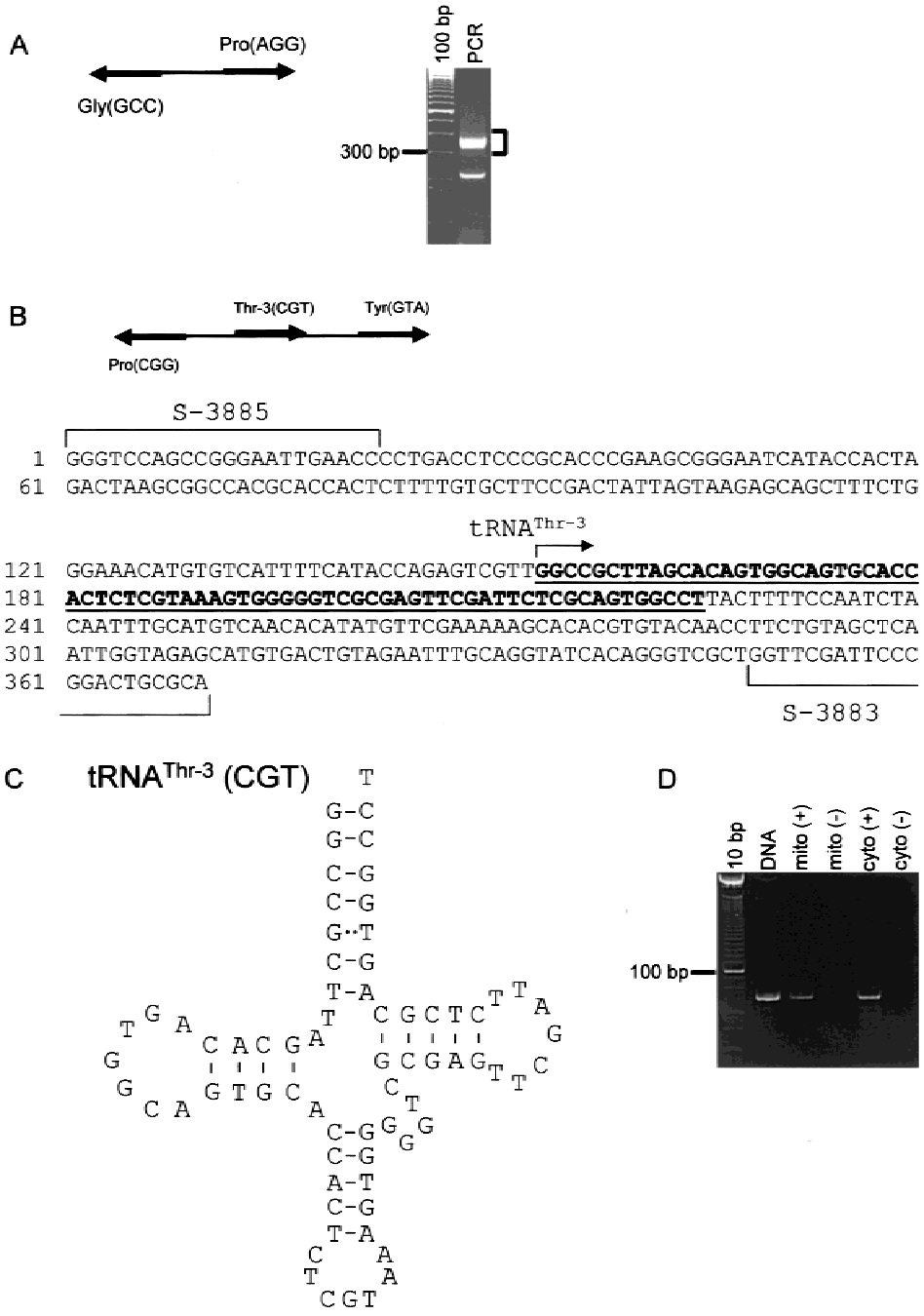

FIGURE 7. PCR amplification of tRNA genes based on conserved motifs. **A**: Left side: A diagram of a 216-bp fragment from *Leptomonas* showing the tail-to-tail organization of two tRNA genes. Right side: *L. tarentolae* genomic DNA was used as a template for PCR using oligonucleotides S-3883 and S-3885 (based on the *Leptomonas* $tRNA^{Pro}$ and $tRNA^{Gly}$ sequences); the bracket indicates the PCR product that was cloned and sequenced. **B**: Top: A diagram of the 350-bp fragment obtained by PCR amplification of *Leishmania* genomic DNA using oligonucleotides S-3883 and S-3885. Bottom: The nucleotide sequence of the PCR product from **A**; primer sequences are indicated at each end of the sequence; the tRNA-coding sequence is shown in bold, underlined text. **C**: The predicted clover-leaf structure of the $tRNA^{Thr-3}$(CGT) from the nucleotide sequence shown in **B**. **D**: RT-PCR of $tRNA^{Thr-3}$(CGT) shows that this tRNA gene is expressed. Shown are a 10-bp ladder (10 bp) with the 100-bp band indicated; PCR amplification with genomic DNA as a template (DNA); RT-PCR amplification from mitochondrial and cytosolic RNA (mito and cyto) with and without reverse transcriptase (as indicated by + and −).

The most striking aspect of this analysis is that there is substantial quantitative variation of the partitioning among the tRNAs classified as mainly cytosolic and mainly mitochondrial, and that the majority of the tRNAs examined show a localization ratio of approximately 1. The reasons for the preferential importation of some tRNAs into the mitochondrion of trypanosomatids is still unknown. In other organisms, the abundance of

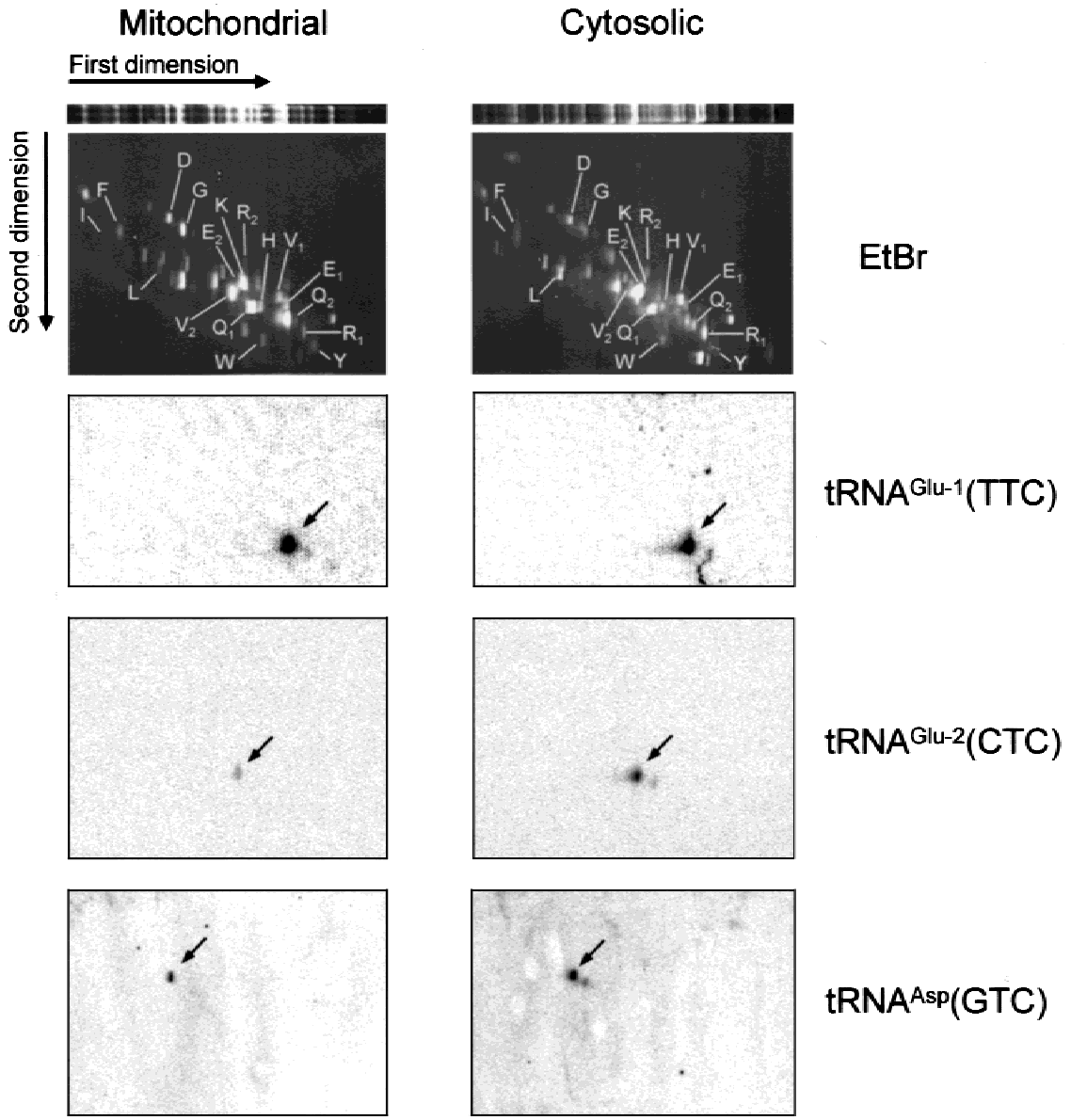

FIGURE 8. Separation of the tRNAs of *L. tarentolae* by two-dimensional PAGE. Seven micrograms of either the mitochondrial (left) or cytosolic (right) tRNA were separated. The identification of 17 spots, as determined by northern analysis, is indicated on the ethidium-stained gel (EtBr). Letters refer to the standard amino acid one-letter code. Also shown are the results of the northern analysis of three of the newly identified tRNAs described in this study (arrows indicate the specific signals from northern analysis).

specific tRNAs is directly correlated to the frequency of the cognate codon (Ikemura, 1985; Yamao et al., 1991; Dong et al., 1996). Determining if tRNA localization in *L. tarentolae* is related to codon usage will require a comprehensive list of tRNAs along with their subcellular distributions and expression levels, as well as information regarding expression levels of mRNAs in the nucleus and the mitochondrion. The data presented in this study should provide a foundation for studying the signals involved in the subcellular partitioning of tRNAs in *Leishmania*.

MATERIALS AND METHODS

Cell culture and fractionation

L. tarentolae cells were grown at 27 °C in brain/heart infusion (Difco) medium supplemented with 10 μg/mL hemin (Cal Biochem). Mitochondria were prepared by hypotonic cell breakage and subsequent purification in a 20–35% Renografin (Bracco) gradient (Braly et al., 1974). A cytosolic fraction was obtained by rupturing cells in a Stansted Cell Disruptor in a solution containing 0.5 M hexylene glycol, 1 mM PIPES, pH 7.4, and 1 mM $CaCl_2$, then clearing the lysate by two successive

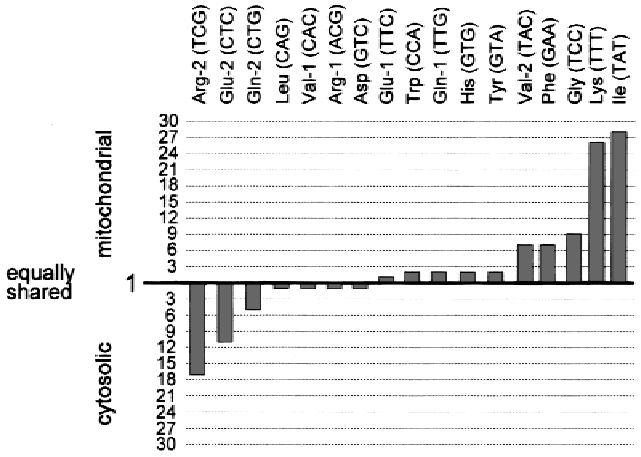

FIGURE 9. Distribution of the tRNAs of *L. tarentolae*. The localization ratio (calculated as described in Materials and Methods) of each tRNA, as labeled at the top of the graph, was calculated and plotted. A localization ratio of 1 indicates that a tRNA is equally shared between the mitochondrial and cytosolic tRNA fractions; bars graphed above the 1 line indicate the tRNA was more abundant in the mitochondrial RNA fraction; bars graphed below the 1 line indicate that the tRNA was more abundant in the cytosolic RNA fraction.

centrifugations (Kapushoc et al., 2000). Analyses of the RNA from the cell fractions have indicated that these methods yield mitochondrial and cytosolic RNA with less than 1% cross contamination (Lima & Simpson., 1996; Kapushoc et al., 2000).

Oligonucleotides

The following oligonucleotides were used for NORTHERN analysis of the indicated tRNAs:

S-3433 (Gln-1) 5′-TTTTCGGATTCAAAGTCCGAAGTG-3′;
S-3434 (Gln-2) 5′-TTATCGGATTCAGAGTCCGAGGTG-3′;
S-3435 (Gly) 5′-TCACCCGCTTGGAAGGCGGGGATC-3′;
S-3436 (Leu) 5′-CCTCCAGGGGAGATCACGACCTGAA CGT-3′;
S-3437 (Val-1) 5′-ATCTCTCGCGTGTGAGGCGAATGTC-3′;
S-3438 (Val-2) 5′-ACGTTCTGCGTGTAAAGCAGACATC-3′;
S-3439 (His) 5′-TATTCAGAGCCACAATCTGATGTT-3′;
S-3440 (Phe) 5′-TTCAGATCTTCAGTCTGACGCT-3′;
S-3441 (Lys) 5′-ACGAGGTTAAAAGCCACGCGCT-3′;
S-3442 (Trp) 5′-CCTGGATTTGGAATCCAATGCT-3′;
S-3443 (Thr-2) 5′-CCTGGTTTACAAGACCAGTGCA-3′;
S-3444 (Ile) 5′-TCCGGTTCATAAGACCAGCGTC-3′;
S-3445 (Arg-1) 5′-CTCTGATCCGTAGTCAGATGCT-3′;
S-3446 (Arg-2) 5′-CTCAGATCCGAAGTCTGATGCG-3′;
S-3447 (Thr-1) 5′-TCCGTCTTACTAGGACGGCGCT-3′;
S-3448 (Tyr) 5′-CTGTGATCTACAGTCACATGCT-3′;
S-3226 (Glu-1) 5′-CTCCGATACCGGGAATCCAAC-3′
S-4001 (Glu-2) 5′-CTAGCGGGTGAGAGCCGCTTGTT-3′;
S-3977 (Asp) 5′-CTTCCCGGCCGGGAAATGAACCCG GGT-3′
S-4003 (Thr-3) 5′-CCCCACTTTACGAGAGTGGTGCA-3′.

The following oligonucleotides were used for RT-PCR of new tRNAs: S-3121, 5′-TTGAATTCGCATTGAGCACCTGC TTTTTTTTTTTTTTTTTTGG-3′, was used for RT of polyadenylated tRNA; S-3124, 5′-pTCTTTAGTGAGGGTTAATT GCCddA-3′ (containing a terminal 5′-phosphate and a terminal 2′,3′-dideoxyadenosine), was the anchor oligonucleotide ligated to the resulting cDNA (the oligonucleotide was synthesized with a 5′-phosphate, and the dideoxyadenosine was added using terminal deoxynucleotidyl transferase (Life Technologies) according to the manufacturer's directions); S-3123, 5′-TTGAATTCGCATTGAGCACCTGC-3′, and S-3125, 5′-GGCAATTAACCCTCACTAAAG-3′, were used for PCR amplification of the double-tagged cDNA.

The following oligonucleotides were used to PCR amplify the $tRNA^{Thr-3}$ fragment (Fig. 7) described in this study: S-3883, 5′-TGCGCAGTCCGGGAATCGAACC-3′, and S-3885, 5′-GGGTCCAGCCGGGAATTGAACC-3′ (based on GenBank LSE245951). Oligonucleotides S-5010, 5′-AGGCCACTGCG AGAATC-3′, and S-5011 5′-GGCCGCTTAGCACAGTGG-3′, were used to RT-PCR amplify the $tRNA^{Thr-3}$(CGT) transcript.

Oligonucleotide S-4012, 5′-TTCTCGGTAGTATAGTGGTT AG-3′, was used as a forward primer for PCR amplification $tRNA^{Asp}$ (with oligonucleotide S-3977).

Polyadenylation of tRNA

The tRNAs (entire tRNA fractions, or individual tRNAs) were polyadenylated as previously described (Sampson & Saks, 1996). The RNA was incubated in a 100-μL reaction containing 20 mM Tris, pH 7, 50 mM KCl, 0.7 mM $MnCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, 10% glycerol, 500 μM ATP, and 1,700 U of yeast poly(A) polymerase (Pharmacia). After incubation at 37 °C for 45 min, 30 μL of 5× *Escherichia coli* supplement buffer (200 mM Tris, 1 M NaCl, 25 mM $MgCl_2$, 12.5 mM $MnCl_2$), 15 μL of 5 mM ATP, and 10 U of *E. coli* poly(A) polymerase (Life Technologies) were added. After further incubation at 37 °C for 45 min, RNA was extracted with phenol and recovered by ethanol precipitation.

Reverse transcription of polyadenylated tRNA

Polyadenylated tRNA was reverse transcribed with Superscript II RNase H^- reverse transcriptase (Life Technologies) using oligonucleotide S-3121 according to the manufacturer's directions. The reaction was initiated at 45 °C (to accommodate the poly(A) target) for 15 min, then the temperature was increased to 50 °C for 35 min to better transcribe through the high secondary structure of the tRNA. After reverse transcription, the RNA was removed by addition of RNase H and RNase A, and the cDNA was extracted with phenol and recovered by ethanol precipitation. The cDNA was then purified from an 8 M urea 6% polyacrylamide gel.

Ligation of cDNA to the anchor oligonucleotide

The anchor oligonucleotide (S-3124, containing a 5′-end phosphate, and blocked at the 3′ end with 2′,3′ dideoxyadenosine) was ligated to the gel-isolated cDNA as previously described (Edwards et al., 1991; Troutt et al., 1992; Morse & Bass, 1997; Williams et al., 2000). Each 50-μL reaction contained cDNA, 30 pmol S-3124, 50 mM Tris, pH 8,

10 mM $MgCl_2$, 10 μg/mL BSA, 1 mM hexaminecobalt (III) chloride, 20 μM ATP, 12.5% PEG 8000, and 18 U of T4 RNA ligase. The ligation reaction was performed at room temperature for 16 h, and the DNA was then recovered by ethanol precipitation.

PCR amplification of double-tagged cDNAs

The double-tagged cDNA was PCR amplified in a 50-μL reaction containing 40 pmol of each oligonucleotide (S-3123 and S-3125), and 1× PCR buffer (Promega) with 2 mM $MgCl_2$, 500 μmol each dNTP, and 5 U of *Taq* DNA polymerase. Thermal cycling profile was as follows: 4 min at 94 °C; 5 cycles of 94 °C 30 s, 40 °C 1 min, 55 °C 1 min; 30 cycles of 94 °C 30 s, 50 °C 1 min, 70 °C 1 min; 10 min at 72 °C. PCR products were gel purified from agarose gels as previously described (Kapushoc et al., 2000).

Cloning and sequencing of PCR products and genomic fragment

Gel-isolated PCR products were cloned using the pCR-2.1-TOPO cloning kit (Invitrogen). Genomic fragments were cloned into pBluescript SK (Stratagene) that had been digested with the appropriate restriction enzymes and treated with CIAP (Life Technologies). Plasmids were sequenced using Sequenase version 2.0 (United States Biochemical) or using the ABI PRISM Dye Terminator Cycle Sequencing kit.

RNA isolation, two-dimensional gel electrophoresis, and northern analysis

RNA was isolated from the mitochondrial and cytosolic fractions by the guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi, 1987). The tRNAs (either the entire tRNA fraction from a one-dimensional gel, or individual tRNA spots from a two-dimensional gel) were eluted from polyacrylamide gels overnight at 4 °C using 300 mM sodium acetate, followed by precipitation with ethanol. For two-dimensional analysis of tRNAs, the RNA was separated in the first dimension using 15% polyacrylamide with 4 M urea, followed by separation in the second dimension using 20% polyacrylamide with 8 M urea (Kapushoc et al., 2000). For northern analysis of the individual tRNAs, the RNA was electroblotted to a Zeta-probe (BioRad) nylon membrane and probed with the appropriate [^{32}P] 5′-end-labeled oligo. Hybridization, washing, and stripping of the probes were done according to the manufacturer's directions. Hybridized membranes were exposed to a phosphorimager screen, imaged with a Storm Phosphorimager, and signals were analyzed with ImageQuant software (Molecular Dynamics).

Quantitative determination of tRNA localization

We have expressed the compartmental localization of each tRNA as the ratio of the signal from the mitochondrial RNA northern to the signal from the cytosolic RNA northern:

$$\text{mito signal/cyto signal} = \text{localization ratio}.$$

If a tRNA has an equal signal in each northern, the ratio is 1; if the tRNA is more abundant in the mitochondrial RNA fraction the ratio is greater than 1; and if the tRNA is more abundant in the cytosolic RNA the ratio is less than 1. For ease of plotting (Fig. 9), if the ratio is less than 1 (i.e., a "cytosolic" tRNA), we have taken the reciprocal of the ratio and graphed it below the equally shared line labeled "1" (e.g., a 0.2 ratio is plotted as a 5 on the cytosolic (bottom) portion of the graph).

Note: Nucleotide sequence data reported in this article have been submitted to GenBank with accession numbers AF409071–AF409074.

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