THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 48, Issue of December 1, pp. 37907–37914, 2000
Printed in U.S.A.
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End Processing Precedes Mitochondrial Importation and Editing of tRNAs in *Leishmania tarentolae**

Received for publication, August 28, 2000
Published, JBC Papers in Press, September 18, 2000, DOI 10.1074/jbc.M007838200

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All mitochondrial tRNAs in *Leishmania tarentolae* are encoded in the nuclear genome and imported into the mitochondrion from the cytosol. One imported tRNA (tRNA^{Trp}) is edited by a C to U modification at the first position of the anticodon. To determine the *in vivo* substrates for mitochondrial tRNA importation as well as tRNA editing, we examined the subcellular localization and extent of 5′- and 3′-end maturation of tRNA^{Trp}(CCA), tRNA^{Ile}(UAU), tRNA^{Gln}(CUG), tRNA^{Lys}(UUU), and tRNA^{Val}-(CAC). Nuclear, cytosolic, and mitochondrial fractions were obtained with little cross-contamination, as determined by Northern analysis of specific marker RNAs. tRNA^{Gln} was mainly cytosolic in localization; tRNA^{Ile} and tRNA^{Lys} were mainly mitochondrial; and tRNA^{Trp} and tRNA^{Val} were shared between the two compartments. 5′- and 3′-extended precursors of all five tRNAs were present only in the nuclear fraction, suggesting that the mature tRNAs represent the *in vivo* substrates for importation into the mitochondrion. Consistent with this model, T7-transcribed mature tRNA^{Ile} underwent importation *in vitro* into isolated mitochondria more efficiently than 5′-extended precursor tRNA^{Ile}. 5′-Extended precursor tRNA^{Trp} was found to be unedited, which is consistent with a mitochondrial localization of this editing reaction. T7-transcribed unedited tRNA^{Trp} was imported *in vitro* more efficiently than edited tRNA^{Trp}, suggesting the presence of importation determinants in the anticodon.

Targeting of one or more nucleus-encoded tRNAs to the mitochondrion occurs in a variety of organisms and allows mitochondrial protein synthesis to proceed in cells in which these tRNAs are not encoded in the mitochondrial genome. Importation of tRNAs into mitochondria has been studied in yeast (1), ciliates (2, 3), plants (4), and trypanosomatids (5–9) using both *in vivo* and *in vitro* techniques. In no case is the mechanism of importation of RNA into mitochondria fully understood, and there appear to be differences in the importation process in different organisms. The trypanosomatids *Leishmania tarentolae* and *Trypanosoma brucei* represent an extreme situation in which no tRNAs are encoded in the mitochondrial genome; and therefore, all tRNAs for mitochondrial translation must be encoded in the nucleus and imported from the cytosol (10, 11).

Targeting of specific tRNAs to the mitochondrion in trypanosomatids has been studied *in vivo* by transfection techniques (5) and *in vitro* by importation into isolated mitochondria (12–16). The specificity of targeting as well as the mechanism of transport are still obscure, but the specificity appears to involve, at least in part, the tertiary structure of the RNA molecule (16).

It was previously proposed, on the basis of the detection of precursor tRNAs in *T. brucei* mitochondria, that the substrate for importation is a 5′-precursor species that is processed by an RNase P-like activity within the organelle (17). However, Aphasizhev *et al.* (18) could not obtain any evidence for the existence of such mitochondrion-localized precursors in *L. tarentolae* or *T. brucei*. In addition, the presence of a genomic 5′-flanking sequence was not required for mitochondrial targeting of tRNA^{Ile} in *L. tarentolae* (5) or for mitochondrial targeting of tRNA^{Tyr} in *T. brucei* (8). However, evidence was recently presented that a dicistronic tRNA transcript in *T. brucei* is the preferred substrate for importation into isolated mitochondria, but no evidence was presented for intramitochondrial processing of this transcript (19, 20).

To investigate the question of the nature of the *in vivo* substrate for tRNA importation into the mitochondrion, we have used RT-PCR[1] to analyze the intracellular localization of 5′- and 3′-end processing and an *in vitro* assay to analyze the importation properties of several specific tRNAs in *L. tarentolae*. We have also investigated the subcellular localization of the C to U editing of the anticodon of the tRNA^{Trp}.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Fractionation, and RNA Isolation—L. tarentolae cells were grown at 27 °C in brain/heart infusion medium (Difco) supplemented with 10 μg/ml hemin (Calbiochem). Mitochondria were prepared from cultures at $\sim 1 \times 10^8$ cells/ml by the hypotonic breakage method as described previously (21). The mitochondria were isolated in a 20–35% Renografin (Bracco) gradient. Isolated mitochondria were treated with micrococcal nuclease to remove any RNA bound to the outside of the vesicles.

Nuclei were prepared from cultures at $\sim 5 \times 10^7$ cells/ml. Cells were washed in 0.15 M NaCl and 0.02 M $NaPO_4$ (pH 7.4); resuspended in 0.5 M hexylene glycol (Sigma), 1 mM PIPES (pH 7.4), and 1 mM $CaCl_2$ (22); and broken using a Stansted Fluid Power apparatus at 60 p.s.i. A portion of this cell lysate was cleared by two successive centrifugations at $15,000 \times g$ for 25 min each to obtain the cytosolic cell fraction. Nuclei were isolated from the cell lysate in a density gradient created by

* This work was supported in part by a Human Frontier Science Program grant (to L. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported in part by Institutional National Research Service Award T32 GM08375 from the United States Public Health Service (UCLA Biotechnology Training Program).

‖ Supported in part by a National Science Foundation Graduate Research Fellowship.

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[1] The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; snRNA, small nuclear RNA; RACE, rapid amplification of cDNA ends; kb, kilobases.

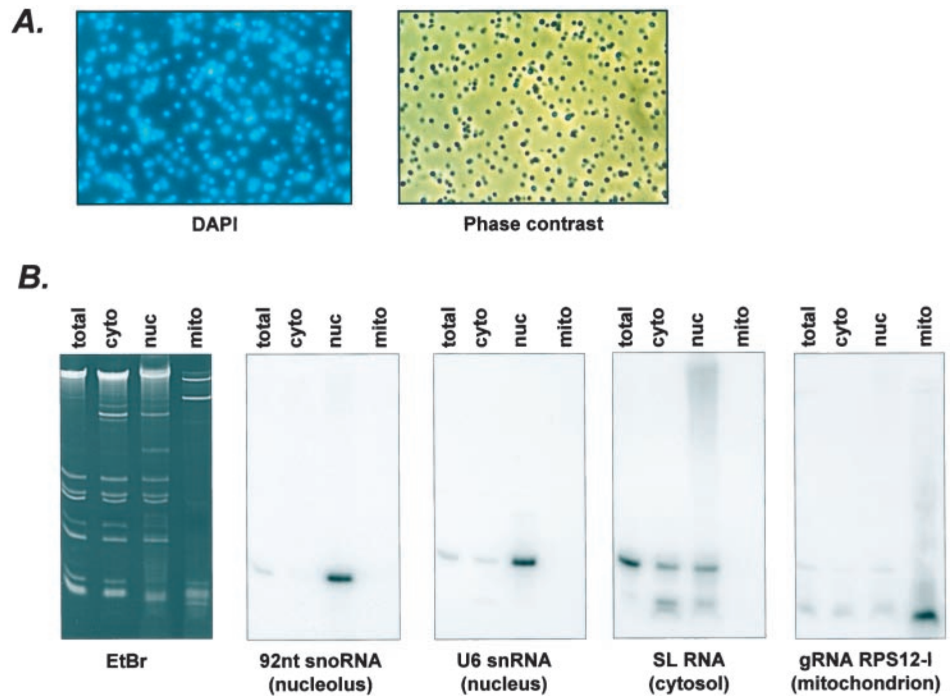


FIG. 1. **Separation of *L. tarentolae* into nuclear, cytosolic, and mitochondrial fractions.** *A*, microphotography of isolated nuclei. The purified nuclei were photographed at 1000× under both phase-contrast and 4,6-diamidino-2-phenylindole (*DAPI*)-stained (with ultraviolet light) conditions (as indicated). *B*, Northern analysis of RNA isolated from different cell fractions. 1.5 μg of RNA isolated from whole cells (*total*), the cytosolic fraction (*cyto*), purified nuclei (*nuc*), or purified mitochondria (*mito*) were separated on an 8 M urea and 6% acrylamide gel. The ethidium bromide-stained gel is shown with the results of Northern hybridizations using oligonucleotide probes specific for several marker RNAs (as indicated). *U6 snRNA*, U6 small nuclear RNA; *SL RNA*, spliced leader RNA; *gRNA RPS12-I*, RPS12 block I guide RNA.

centrifugation of 35% Percoll (Amersham Pharmacia Biotech) at 60,000 × *g* for 35 min. Each step of the cell fractionation was monitored by phase and fluorescence microscopy. RNA was isolated from whole cells and from each cell fraction using the guanidinium thiocyanate/phenol/chloroform extraction method (23).

Northern Analysis—RNA (1.5 μg) from each cell fraction was separated on an 8 M urea and 6% acrylamide gel. The gel was stained with ethidium bromide for RNA visualization and photography, and the RNA was transferred to Zeta-Probe membranes (Bio-Rad) according to the manufacturer's directions. The membranes were hybridized with the appropriate oligonucleotides, which were 5′-end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Life Technologies, Inc.).

Two-dimensional Gel Electrophoresis—7 μg of mitochondrial and cytosolic tRNAs were resuspended in 20 μl of 4 M urea and electrophoresed in the first dimension on a 4 M urea and 15% polyacrylamide gel (200 × 350 × 0.75 mm) at 750 V for 24 h at room temperature. The gel was stained with ethidium bromide, and the tRNA portion of the gel was excised and layered on top of a second dimension 8 M urea and 20% polyacrylamide gel (180 × 250 × 0.75 mm). The second dimension was electrophoresed at 400 V for 48 h at room temperature. The separated tRNAs were visualized by ethidium bromide staining for photography and transferred to a Zeta-Probe membrane for Northern analysis as described above.

Clamped Homogeneous Electric Field Electrophoresis—*L. tarentolae* genomic DNA blocks were prepared as describe previously (24, 25) and separated on 0.5× Tris borate/EDTA-containing 1.5% agarose using the CHEF-DR II apparatus (Bio-Rad). The chromosome bands were visualized by ethidium bromide staining, and the gel was photographed using a C-80 UV Darkroom system (Ultra-Violet Products). The DNA was transferred to Nytran Plus membranes (Schleicher & Schüll), which were hybridized with the different tRNA PCR products labeled with [α-^{32}P]dATP using the Prime-It II random primer labeling kit (Stratagene).

Oligonucleotides—The following oligonucleotides were used for Northern analysis of RNA. S-3434 (5′-TTATCGGATTCAGAGTCCGAGGTG-3′) was used to detect tRNA^{Gln}(CUG); S-3442 (5′-CCTGGATTTGGAATCCAATGCT-3′) was used to detect tRNA^{Trp}(CCA); S-3444 (5′-TCCGGTTCATAAGACCAGCGTC-3′) was used to detect tRNA^{Ile}(UAU); S-3443 (5′-ACGAGGTTAAAAGCCACGCGCT-3′) was used to detect tRNA^{Lys}(UUU); S-3437 (5′-ATCTCTCGCGTGTGAGGCGAATGTC-3′) was used to detect tRNA^{Val}(CAC); S-3313 (5′-CAACGTCCATCTGCGACGGCTTTA-3′) was used to detect the 92-nucleotide small nucleolar RNA; U6-1 (5′-TCTTCACTGTTGAATTTCC-3′) was used to detect the U6 snRNA; S-3315 (5′-GTTCCGGAAGTTTCGCATAC-3′) was used to detect the spliced leader RNA; and S-3316 (5′-GTCTTCCTCTGAATGCGTAAGCG-3′) was used to detect the RPS12-I guide RNA.

The following oligonucleotides (based on GenBank™/EBI accession number L20948 (33)) were used for RT-PCR of mature and precursor tRNA^{Trp}. S-3213 (5′-ATCGAAAATGCGCTGTGACTTGTGG-3′) was used for amplification of the tRNA^{Trp} 5′-leader; S-2820 (5′-AGCTCAGTGGTAGAGCATTGG-3′) was used for amplification of the mature tRNA^{Trp} 5′-end; S-3215 (5′-CCAAAAAGGGCCCCCAGCGCGAAAACC-3′) was used for amplification of the tRNA^{Trp} 3′-trailer; and S-2819 (5′-TGAGAGCTGCAGGGATTGAAC-3′) was used for amplification of the mature tRNA^{Trp} 3′-end.

The following oligonucleotides (based on GenBank™/EBI accession number L20948 (33)) were used for RT-PCR of mature and precursor tRNA^{Ile}: S-3478 (5′-CCTACATCCATATTCGCAGTATGT-3′) was used for amplification of the tRNA^{Ile} 5′-leader; S-3479 (5′-GCTCCCGTGGTCTAGTTGGTTAGG-3′) was used for amplification of the mature tRNA^{Ile} 5′-end; S-3480 (5′-TTGGGGATTTTGGGGGCGGAAAAG-3′) was used for amplification of the tRNA^{Ile} 3′-trailer; and S-3385 (5′-GGCTCGAACCCGCGACATCCGGTTC-3′) was used for amplification of the mature tRNA^{Ile} 3′-end.

The following oligonucleotides (based on GenBank™/EBI accession number X68207 (34)) were used for RT-PCR of mature and precursor tRNA^{Gln}. S-3475 (5′-TCACCTTCATCATCCTTGTATGAT-3′) was used for amplification of the tRNA^{Gln} 5′-leader; S-3476 (5′-GCTCCTATAGTGTAGCGGTTATCA-3′) was used for amplification of the mature tRNA^{Gln} 5′-end; S-3477 (5′-GCCGCTTTTTGGTGGGGGGGTAAA-3′) was used for amplification of the tRNA^{Gln} 3′-trailer; and S-3387 (5′-GGACTCGAACCAGGGTTATCGGATT-3′) was used for amplification of the mature tRNA^{Gln} 3′-end.

The following oligonucleotides (based on based on GenBank™/EBI accession number L20948 (33)) were used for RT-PCR of mature and precursor tRNA^{Lys}. S-3864 (5′-TGGAGTAGCAATACGAATTCC-3′) was used for amplification of the tRNA^{Lys} 5′-leader; S-3865 (5′-GCACTTCTAGCTCAGTTGGTAG-3′) was used for amplification of the mature tRNA^{Lys} 5′-end; and S-3866 (5′-CGCACTCCGTGGGGCTCGAAC-3′) was used for amplification of the mature tRNA^{Lys} 3′-end.

The following oligonucleotides (based on GenBank™/EBI accession number AF016249 (37)) were used for RT-PCR of mature and precursor tRNA^{Val}. S-3861 (5′-TTGTAGCTCACATCCAGTAGC-3′) was used for amplification of the tRNA^{Val} 5′-leader; S-3862 (5′-GCGATGGTCGTCTAGTGGTTAG-3′) was used for amplification of the mature tRNA^{Val} 5′-end; and S-3863 (5′-TACGACGGGCGGGGATTGAAC-3′) was used for amplification of the mature tRNA^{Val} 3′-end.

The following oligonucleotides were used for 3′-RACE of tRNA^{Trp}. S-3122 (5′-TTGAATTCGCATTGAGCACCTGCTTTTTTTTTTTTTTTTTT-3′) was used for reverse transcription of polyadenylated RNA, and S-3123 (5′-TTGAATTCGCATTGAGCACCTGC-3′) was used for PCR amplification of the resulting cDNA.

RT-PCR, Cloning, and Sequencing—For reverse transcription reactions, 40 pmol of the appropriate oligonucleotide primer were combined with 2 μg of each RNA in a 20-μl reaction using 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) following the directions of the manufacturer. Identical reactions were performed without the addition of Superscript II for the no-reverse transcriptase control. 1 μl of each reverse transcription reaction was used as a template for PCR amplification. Each 50 μl of PCR contained 40 pmol of each of the appropriate oligonucleotide primers and 1× PCR buffer (Promega) with 2 mM $MgCl_2$, 500 μM each dNTP, and 5 units of *Taq* DNA polymerase. Thermal cycling was performed with an initial denaturation at 95 °C for 4 min, followed by 30 cycles of PCR (95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 10 min. The PCR products were analyzed on a 2.5% Metaphor-agarose gel (FMC Corp. BioProducts). PCR products were cloned using the pCR-2.1-TOPO cloning kit (Invitrogen) and sequenced using Sequenase Version 2.0 (U. S. Biochemical Corp.).

HinfI Digestion of RT-PCR Products—Precursor $tRNA^{Trp}$ RT-PCR products were purified from a 2.5% Metaphor-agarose gel to separate the PCR product from unincorporated primers and nucleotides. The PCR products were digested with *Hin*fI. After digestion, the reactions were extracted with phenol/chloroform, ethanol-precipitated, and analyzed on a 2.5% Metaphor-agarose gel.

Assay of in Vitro Importation of RNA into Isolated Mitochondria—The *in vitro* RNA importation assay (16) was performed in a 20-μl reaction volume containing 50,000 cpm radioactively labeled RNA, 1 mg of mitochondria (~40 μg of protein), 1 mM ATP, 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.63 mM creatine phosphate, and 22.5 mg/ml creatine phosphokinase (5, 12, 14, 15). After incubation at 27 °C for 5–30 min, 100 units of micrococcal nuclease (Roche Molecular Biochemicals) and 5 mM $CaCl_2$ were added to digest the RNAs that were not imported into the mitochondria. Micrococcal nuclease was then inhibited by the addition of 10 mM EGTA (pH 8). To isolate imported RNAs, the mitochondria were pelleted and suspended in 150 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS and extracted with phenol; and the RNA was precipitated with ethanol. The radioactively labeled RNAs were separated by electrophoresis on a 7 M urea and 8% acrylamide gel. After electrophoresis, the nuclease-protected radioactively labeled RNAs were visualized and quantitated using a Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

3′-RACE Analysis of $tRNA^{Trp}$—To map the 3′-end of $tRNA^{Trp}$ by RT-PCR, 15 μg of RNA from each cell fraction were polyadenylated using a combination of yeast poly(A) polymerase (Amersham Pharmacia Biotech) and *Escherichia coli* poly(A) polymerase (Life Technologies, Inc.) according to previously established protocols (26). The resulting polyadenylated RNA was used as a template for cDNA synthesis in a 20-μl reaction using 200 units of Superscript II and primer S-3122. A 5-μl portion of the cDNA reaction was used as a template in PCR using primers S-2820 and S-3123.

RESULTS

Subcellular Fractionation of L. tarentolae—Several methods exist for the isolation of kinetoplast-mitochondrial fractions from *L. tarentolae* cells with <5% contamination with cytosolic rRNA (5). Cells can be ruptured in hypotonic (21) or isotonic (27) media, and mitochondria can be isolated by isopycnic sedimentation in Renografin (21) or Percoll (28) gradients. The hypotonic rupture/Renografin method yields mitochondrial fractions of the highest purity and yield (5).

These methods cannot, however, be used to obtain nuclear fractions due to the presence of chelating agents, which cause the nuclei to rupture during isolation. We have modified the method of Shapiro and Doxsey (22), which was developed for *T. brucei*, by disrupting cells in the Stansted Fluid Power cell disruption apparatus in the presence of 0.5 M hexylene glycol and $CaCl_2$ and banding nuclei in a Percoll gradient. As shown in the micrograph in Fig. 1*A*, this method produces a high yield of morphologically intact nuclei with essentially no intact cells or contaminating debris visible at the level of light microscopy. Furthermore, since the kinetoplast-mitochondrion remains intact and associated with the flagellum and cell ghost after cell rupture, clarification of the lysate yields a cytosolic cell fraction with little apparent mitochondrial or nuclear contamination.

To ascertain the purity of the subcellular fractions, equal amounts of RNA isolated from each cell fraction were analyzed by gel electrophoresis as shown in Fig. 1*B*. The cytosolic and nuclear fractions show, in addition to tRNA, the predominant

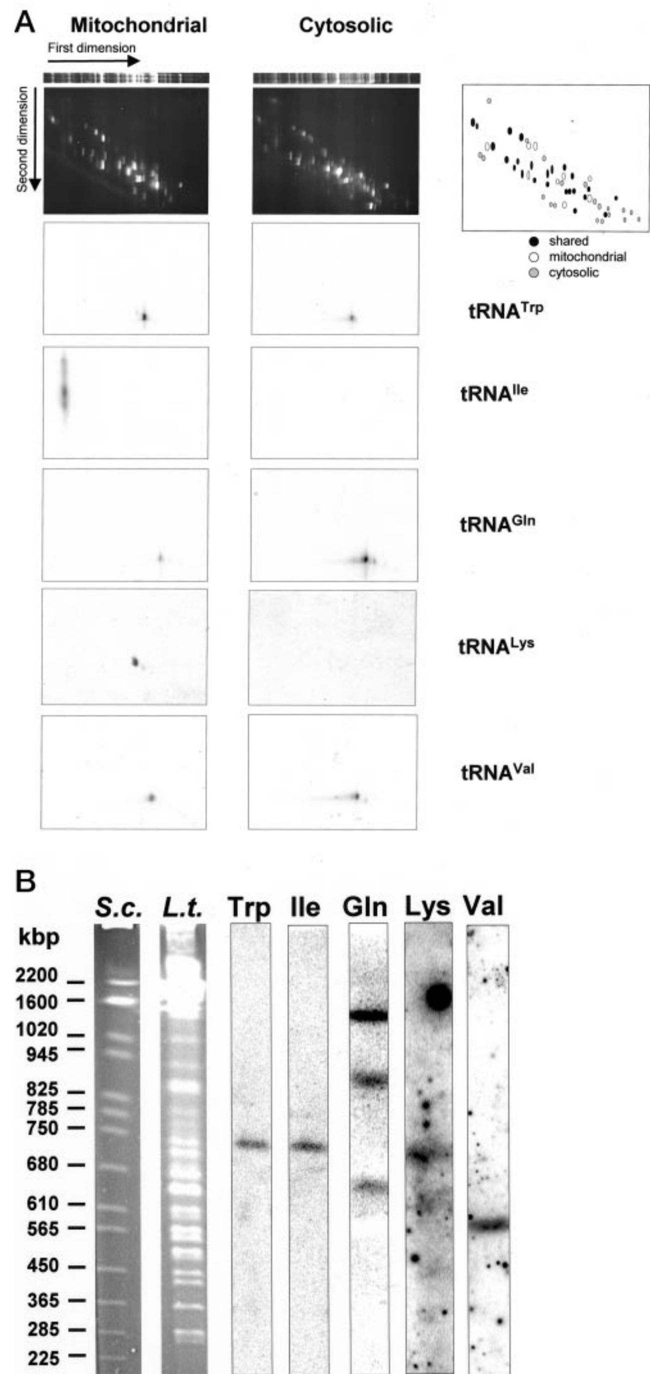


FIG. 2. **Subcellular distribution, separation, and genomic localization of $tRNA^{Trp}$, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Val}$.** *A*, separation of mitochondrial and cytosolic tRNAs by two-dimensional electrophoresis. ~7 μg of tRNA from either the cytosolic or mitochondrial fraction were separated by two-dimensional gel electrophoresis. The ethidium bromide-stained gels are shown (the first dimensions are shown on top) with a schematic diagram indicating the distribution of the individual tRNAs. The results of Northern hybridizations using oligonucleotides specific for each of the five tRNAs (as indicated) to specific tRNA spots are also shown. *B*, Southern blot of a clamped homogeneous electric field gel electrophoresis of *L. tarentolae* chromosomal DNA. *S. cerevisiae* (*S.c.*) chromosomes (with sizes indicated in kilobase pairs (*kbp*) and separated *L. tarentolae* (*L.t.*) chromosomes are shown. The results of Southern hybridizations with the different tRNA gene probes (Trp, Ile, Gln, Lys, and Val) are shown.

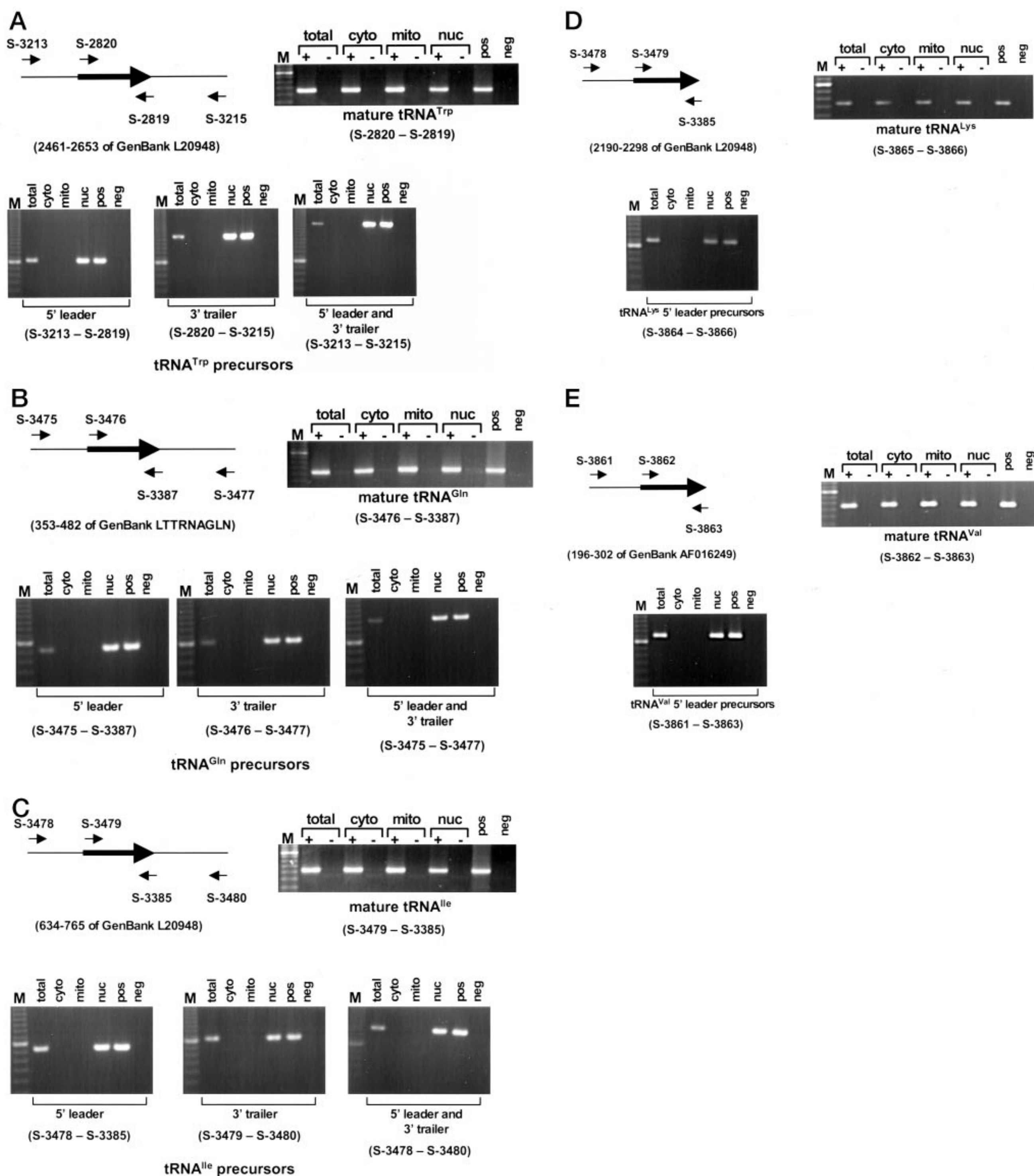
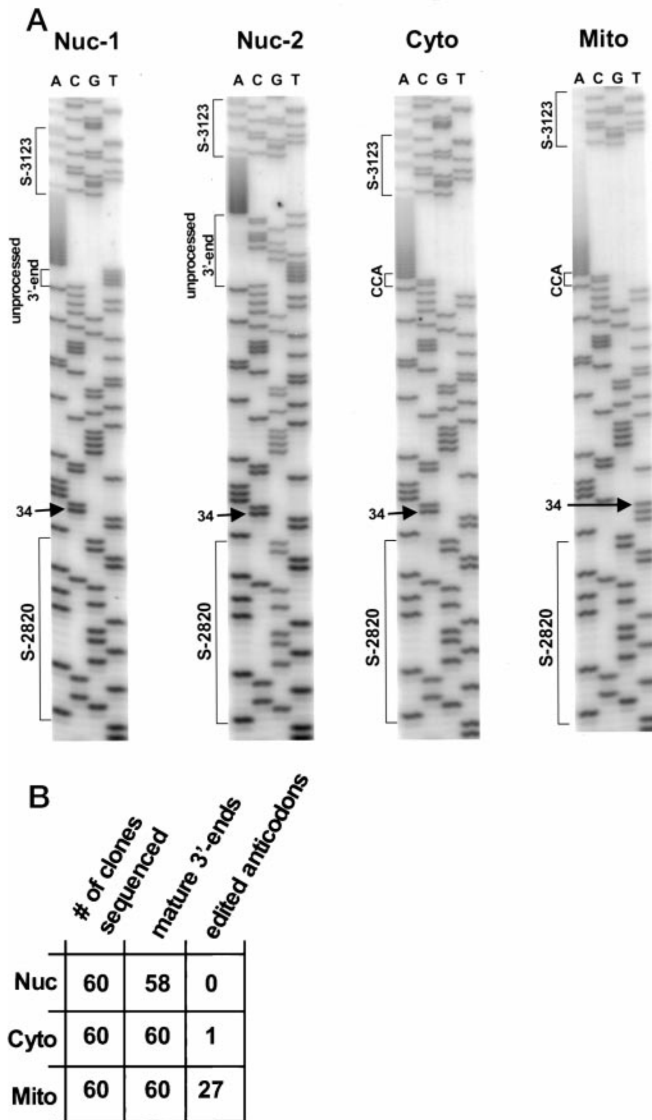


FIG. 3. **RT-PCR detection of pre-tRNAs.** Shown are the results from RT-PCR analysis of $tRNA^{Trp}$ (*A*), $tRNA^{Gln}$ (*B*), $tRNA^{Ile}$ (*C*), $tRNA^{Lys}$ (*D*), and $tRNA^{Val}$ (*E*) from total, cytosolic (*cyto*), mitochondrial (*mito*), and nuclear (*nuc*) RNAs (as indicated). Each panel contains a *schematic* (*upper left panel*) showing the placement of the primers used for RT-PCR. In each gel, the marker lane (*M*) is a 10-base pair ladder (Life Technologies, Inc.). The most intense band is 100 base pairs. *pos* and *neg* are positive and negative PCR controls, respectively. The oligonucleotides used for each PCR are indicated below each gel. A control RT-PCR of the mature tRNA sequence is shown (*upper right panel*), in which reactions were performed with (+) and without (−) reverse transcriptase. The bottom portion of each gel shows the RT-PCR of pre-tRNAs using RNA from each cell fraction (only reactions with reverse transcriptase are shown).

five small rRNAs characteristic of trypanosomatids (29), and the mitochondrial fraction shows only the 9 S and 12 S mitochondrial rRNAs (30) and tRNA. In addition, Northern analysis was performed using hybridization probes for marker RNAs specific for the nucleolus (92-nucleotide small nucleolar RNA) (31), the nucleus (U6 snRNA), and the mitochondrion (RPS12-I guide RNA) (32). The probe for the 92-nucleotide small nucleolar RNA showed specific hybridization to the nuclear fraction. The probe for the U6 snRNA showed a large enrichment in the nuclear fraction with some hybridization to the cytosolic fraction; it is unclear whether the latter represents leakage from the nucleus during cell fractionation or the presence of transient U6 snRNA traversing the cytosol during normal RNA maturation and small nucleolar ribonucleoprotein assembly.



	# of clones sequenced	mature 3′-ends	edited anticodons
Nuc	60	58	0
Cyto	60	60	1
Mito	60	60	27

FIG. 4. **3′-RACE analysis of tRNA^{Trp} shows two unprocessed 3′-ends in the nuclear fraction.** *A*, sequences of representative clones from the 3′-RACE of tRNA^{Trp} from different cell fractions. Sequencing lanes are labeled (*A*, *C*, *G*, *T*). The following are indicated on each sequencing ladder: the sequence of the 5′-PCR primer (S-2820); the sequence of the 3′-PCR primer (S-3123); the nucleotide at position 34 (*arrows*); and the location of the 3′-end of the RNA sequence, either unprocessed (*unprocessed 3′-end*) or processed (*CCA*). The sequences shown are as follows: the clones of unprocessed tRNA^{Trp} from the nuclear fraction (*Nuc-1* and *Nuc-2*), unedited tRNA^{Trp} from the cytosolic fraction (*Cyto*), and edited tRNA^{Trp} from the mitochondrial fraction (*Mito*). *B*, summarization of the data from the 3′-RACE analysis of tRNA^{Trp} from the different cell fractions.

The guide RNA probe showed strong hybridization to the mitochondrial fraction and weak hybridization to the cytosolic and nuclear fractions. The probe for the SL RNA showed hybridization to both the nuclear and cytosolic fractions, but not to the mitochondrial fraction. The fact that the small nucleolar RNA, U6 RNA, and SL RNA probes showed no hybridization to the mitochondrial fraction indicates a high level of purity of this cell fraction, which was confirmed by the guide RNA hybridization results. Quantitation of these blots indicated that the mitochondrial fraction had undetectable levels of nuclear or cytosolic contamination, the cytosolic fraction had 0.3% nuclear contamination and <0.1% mitochondrial contamination, and the nuclear fraction had <0.1% mitochondrial contamination.

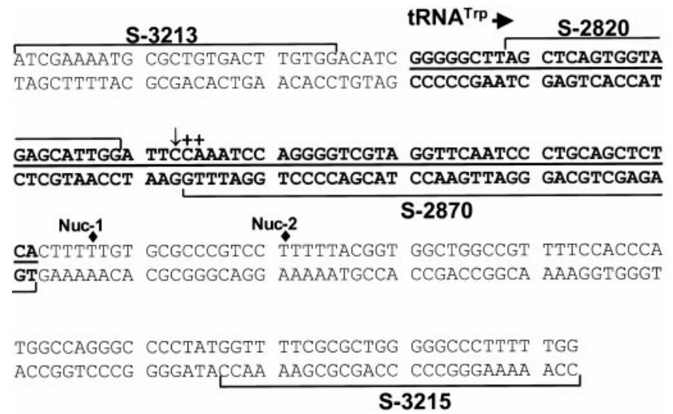


FIG. 5. **The portion of the *L. tarentolae* tRNA gene cluster (nucleotides 2461–2653; GenBank™/EBI accession number L20948) that contains the tRNA^{Trp} gene (33).** The mature tRNA^{Trp} sequence is *underlined* and in *boldface*. The CCA anticodon is indicated (↓ ++), with C^{34} that is edited indicated by the *arrow* (↓). The locations of the unprocessed 3′-ends determined by 3′-RACE (*Nuc-1* and *Nuc-2*; as shown in Fig. 4*A*) are indicated (♦). The locations of the oligonucleotide primers used in this study are indicated.

tRNA^{Trp}(CCA), tRNA^{Ile}(AUA), tRNA^{Gln}(CUG), tRNA^{Lys}(UUU), and tRNA^{Val}(CAC) Are Representative of Three Different Subcellular Distribution Patterns—The intracellular distributions of specific tRNAs were examined using the above cell fractionation procedures. Equal amounts of tRNA from the mitochondrial and cytosolic cell fractions were separated by two-dimensional gel electrophoresis (Fig. 2*A*). Comparison of the patterns of the ethidium bromide-stained gels indicated that ~24 tRNAs are shared between the cytosolic and mitochondrial fractions, 20 are mainly cytosolic, and 9 are mainly mitochondrial. However, it should be noted that compartment-specific nucleotide modifications may alter the mobility of a given tRNA and may lead to the erroneous assignments of import phenotypes on ethidium bromide-stained gels. Therefore, proper assessment of import phenotype must include Northern analysis.

To establish the intracellular distribution of the five specific tRNAs, gels were blotted and hybridized with labeled oligonucleotide probes. The tRNA^{Gln} probe detected a tRNA spot that was more intense in the cytosolic RNA fraction. The tRNA^{Trp} and tRNA^{Val} probes detected single spots of approximately equal intensity in both the cytosolic and mitochondrial RNA fractions. The tRNA^{Ile} probe detected a single spot of low relative intensity in the mitochondrial RNA fraction, and the tRNA^{Lys} probe detected a single spot of high relative intensity in the mitochondrial RNA fraction. From these results, we operationally define the distribution of tRNA^{Trp}(CCA) and tRNA^{Val}(CAC) as a "shared" pattern, the distribution of tRNA^{Gln}(CUG) as a "mainly cytosolic" pattern, and the distribution of tRNA^{Ile}(UAU) and tRNA^{Lys}(UUU) as a "mainly mitochondrial" pattern. These are relative operational designations and do not include corrections for the relative amounts of tRNA in the cytosol and mitochondrion on a per cell basis.

Localization of tRNA Genes—The genes for the tRNAs examined in this study were analyzed by clamped homogeneous electric field gel electrophoresis and Southern blotting (Fig. 2*B*). The tRNA^{Ile} and tRNA^{Trp} genes localized to the same chromosome of ~700 kb. This is consistent with the observations that these two tRNA genes are contained in the same genomic tRNA cluster (33) and are single-copy.[2] The tRNA^{Lys} probe also showed hybridization to the same chromosome of ~700 kb, which is consistent with the fact that this tRNA is in the same gene cluster as tRNA^{Ile} and tRNA^{Trp}. In addition, the

[2] J. D. Alfonzo, S. T. Kapushoc, and L. Simpson, unpublished data.

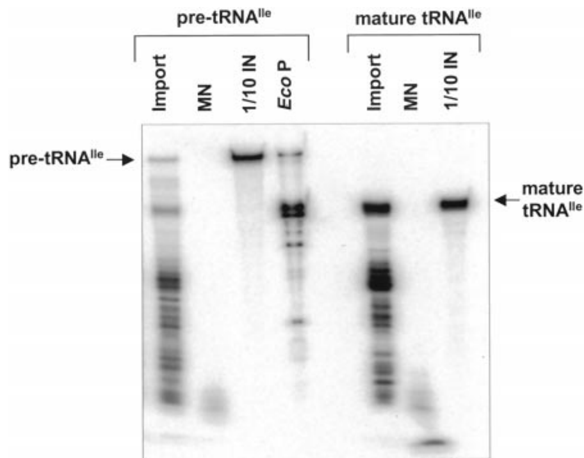


FIG. 6. **Pre-tRNA^{Ile} is not efficiently imported into *L. tarentolae* mitochondria *in vitro*.** Uniformly labeled pre-tRNA^{Ile} and mature tRNA^{Ile} (as indicated) were incubated with isolated mitochondria and then digested with micrococcal nuclease to remove RNA that was not imported (*Import*). The following were included as controls: RNA digested with micrococcal nuclease (*MN*), 10% of the RNA used as input in the importation reaction (*1/10 IN*), and pre-tRNA^{Ile} incubated with *E. coli* RNase P RNA (*Eco P*). The migration of each full-length input RNA is indicated by the corresponding *arrows*.

tRNA^{Lys} probe also showed hybridization to a chromosome of ~680 kb, which may indicate another copy of the same tRNA gene or hybridization of the probe to a different tRNA gene of similar sequence. The probe for tRNA^{Gln} hybridized to three chromosomes of ~1500, 850, and 650 kb. The hybridization pattern of this probe is consistent with the previous observation that this gene is multicopy (34). The tRNA^{Val} probe hybridized to a single chromosome band of ~560 kb.

tRNA Precursors Localize to the Nucleus—To address the question of whether 5′-precursors are *in vivo* substrates for import into the mitochondrion (17), RT-PCR of the mitochondrial and shared tRNAs (tRNA^{Trp}, tRNA^{Ile}, tRNA^{Lys}, and tRNA^{Val}) was performed with RNAs isolated from the nuclear, cytosolic, and mitochondrial cell fractions using primers specific for the flanking genomic sequences (Fig. 3, *A–E*). For comparison, cytosolic tRNA^{Gln} was also assayed. Control amplifications using internal primers yielded products corresponding to the mature tRNA sequences in each case. We found that 5′-precursors of each tRNA could be amplified only from the nuclear fraction, a result consistent with 5′-end processing occurring prior to export from the nucleus, as is the case in other eukaryotic cells (35). Three tRNAs (tRNA^{Trp}, tRNA^{Ile}, tRNA^{Gln}) were assayed in addition by RT-PCR for the subcellular localization of 3′-precursors, and these also localized solely to the nuclear fraction. These data suggest that both 5′- and 3′-end processing of the mitochondrial tRNAs occurs prior to export from the nucleus.

3′-CCA Addition Precedes Nuclear Export—To analyze the subcellular localization of 3′-CCA addition to a tRNA targeted to the mitochondrion, 3′-RACE of tRNA^{Trp} was performed using RNAs isolated from the nuclear, cytosolic, and mitochondrial cell fractions. Of the clones obtained from the nuclear RNA fraction, 58 had mature 3′-CCA ends, and two had unprocessed 3′-ends (Fig. 4*B*). One of the latter sequences terminated at an oligo(T) stretch just downstream of the 3′-end of the mature tRNA (Fig. 4*A*, *Nuc-1*), and the other terminated at another oligo(T) stretch 14 nucleotides farther downstream (*Nuc-2*). The positions of these unprocessed 3′-ends are indicated in Fig. 5 (♦). This lack of precise transcription termination may provide an explanation for the observed PCR amplification of 3′-extended precursor molecules in the tRNA^{Trp} RT-PCR experiment (Fig. 3*A*). Similar analysis of RNAs isolated from the cytosolic and mitochondrial fractions revealed that tRNAs in these fractions contained mature 3′-CCA ends (Fig. 4). This evidence suggests that 3′-CCA addition occurs in the nucleus, even in the case of tRNAs that eventually localize in the mitochondrion.

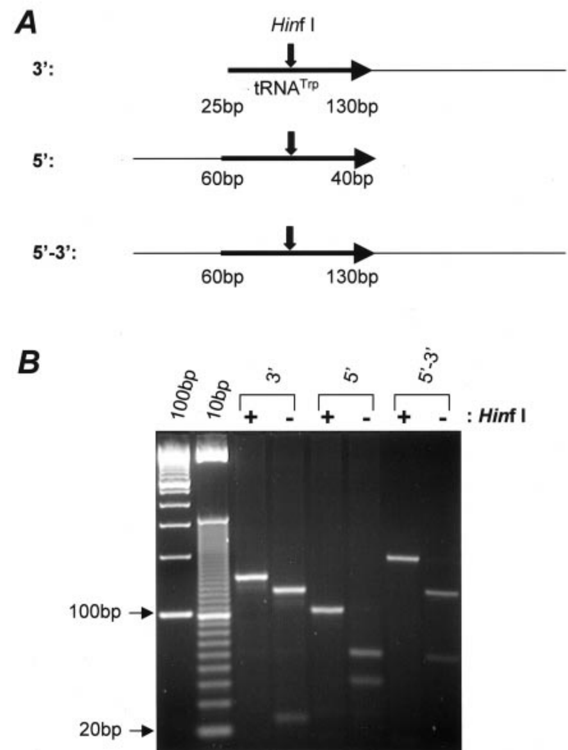


FIG. 7. **Pre-tRNA^{Trp} RT-PCR products are digested with *Hin*fI, indicating that they are not edited at C^{34}.** *A*, schematic diagrams of the precursor RT-PCR products. The precursor sequence is indicated by the *thinner line*, and the position of the mature tRNA^{Trp} sequence is indicated by the *thicker horizontal arrow*. The position of the *Hin*fI site is indicated by the *vertical arrow*. *B*, ethidium bromide-stained gel of the *Hin*fI-digested tRNA^{Trp} RT-PCR products. The marker lanes are 100- and 10-base pair ladders (as indicated), and the positions of the 100- and 20-base pair bands are indicated. Digestion reactions were carried out with (+) and without (−) *Hin*fI. *bp*, base pairs.

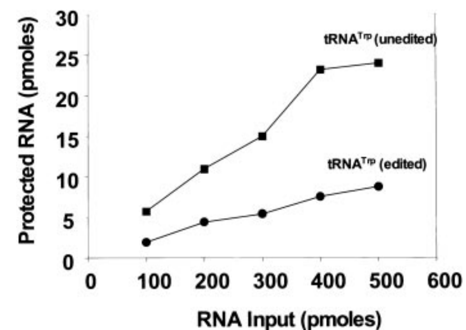


FIG. 8. ***In vitro* importation of unedited tRNA^{Trp} saturates at a higher concentration than edited tRNA^{Trp}.** Increasing amounts of unedited and edited tRNA^{Trp} were incubated with isolated mitochondria, and importation was assayed and quantitated. The *graph* shows the amount of RNA protected when increasing input unedited tRNA^{Trp} (■) or edited tRNA^{Trp} (●) was used.

Mature tRNA^{Ile} Is Imported into Isolated Mitochondria More Efficiently than 5′-Extended Precursor tRNA^{Ile}—The data presented so far are consistent with a model in which the mature tRNA^{Gln}, tRNA^{Ile}, tRNA^{Trp}, tRNA^{Val}, and tRNA^{Lys} molecules are the *in vivo* substrates for importation into the mitochondrion. Rubio *et al.* (16) showed that *in vitro* importation of

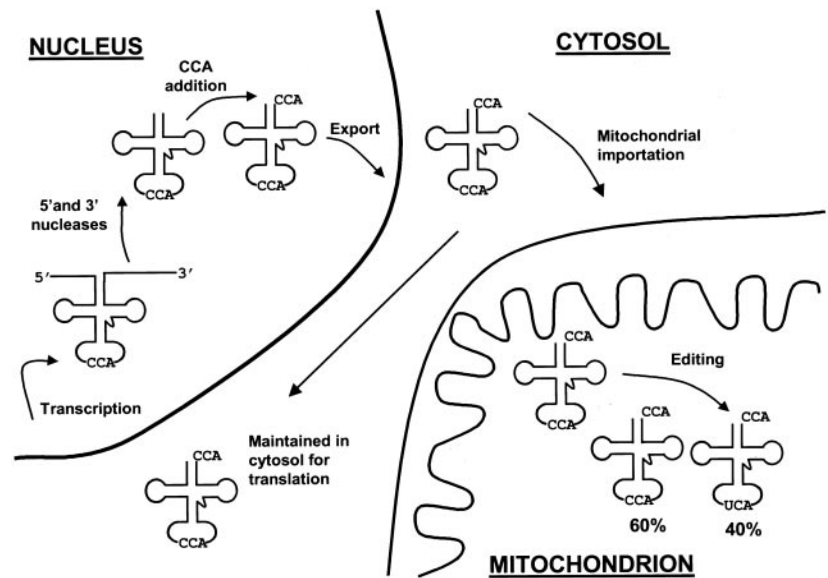


FIG. 9. **Proposed model of *L. tarentolae* tRNA^{Trp} transcription, processing, and editing.** The tRNA is transcribed from the nuclear genome as a precursor with extended 5′- and 3′-ends. The ends are processed, and the 3′-terminal CCA is added to the tRNA in the nucleus. The processed tRNA is then exported to the cytosol. A portion of the exported tRNA is maintained in the cytosol, and a potion is imported into the mitochondrion. Within the mitochondrion, a fraction of the tRNA^{Trp} is edited at the first position of the anticodon (C^{34} to U^{34}).

synthetic tRNAs into isolated mitochondria shows a high level of selectivity that, at least in the case of tRNA^{Ile} and tRNA^{Gln}, is correlated with the *in vivo* subcellular localization. We therefore assayed the *in vitro* importation efficiency of T7-transcribed tRNA^{Ile} with and without a 50-nucleotide 5′-leader. The results shown in Fig. 6 indicate that mature tRNA^{Ile} is the preferred substrate for mitochondrial importation. This selective *in vitro* importation is probably not due to the pre-tRNA folding into an unusual structure since digestion of 5′-extended tRNA^{Ile} with *E. coli* RNase P RNA (44) was shown to cleave the 5′-leader at the expected position (Fig. 6).

Nucleus-localized Precursors of tRNA^{Trp} Are Unedited—The C^{34} to U^{34} editing event of mitochondrial tRNA^{Trp} can be detected by resistance to digestion of the edited cDNA with *Hin*fI since editing destroys a *Hin*fI site (36). As shown in Fig. 7, the amplified cDNAs from each of the tRNA^{Trp} precursors were completely digested by *Hin*fI. The *Hin*fI-digested precursor tRNA^{Trp} RT-PCR products were further analyzed for possible *Hin*fI-resistant molecules by extracting the region of the gel that would contain undigested amplification products and using this as a template for additional cycles of PCR. No amplification products were obtained in this second PCR (data not shown), indicating that the initial RT-PCR of tRNA^{Trp} precursors did not amplify any molecules with edited anticodons. This indicates that editing occurs after 5′- and 3′-end maturation and is consistent with an intramitochondrial localization of this reaction.

T7-transcribed Unedited tRNA^{Trp}(C^{34}CA) Is More Efficiently Imported in Vitro into Isolated Mitochondria Than Edited tRNA^{Trp}(U^{34}CA)—Since mature unedited tRNA^{Trp} appears to represent the *in vivo* substrate for importation into the mitochondrion, we decided to analyze the specificity of the *in vitro* import system for unedited *versus* edited tRNA. A comparison of the *in vitro* importation of T7-transcribed unedited and edited tRNA^{Trp} is shown in Fig. 8. Unedited tRNA^{Trp} reached a higher saturation level than edited tRNA^{Trp}, indicating that the import mechanism can distinguish between these two isoforms.

DISCUSSION

There appear to be at least three overlapping classes of tRNAs in *L. tarentolae* in terms of subcellular localization: 1) mainly cytosolic, 2) mainly mitochondrial, and 3) shared between the two compartments. This is based on two-dimensional gel analysis of cytosolic and mitochondrial tRNAs and on Northern blot analysis of five specific tRNAs: tRNA^{Trp}, tRNA^{Ile}, tRNA^{Gln}, tRNA^{Lys}, and tRNA^{Val}. We showed in this study that tRNA^{Lys} represents a second mitochondrion-localized tRNA in addition to the previously identified tRNA^{Ile}. This differs from the previous result of Suyama and co-workers (33, 37), who classified tRNA^{Lys} as having a shared phenotype.

The signals for mitochondrial targeting are not known, although we have shown previously that swapping the D-arm between mitochondrion-localized tRNA^{Ile} and cytosolic tRNA-^{Gln} reversed the importation phenotypes of these molecules both *in vivo* and *in vitro* (5, 16), suggesting that the tertiary structure of the molecule is involved in the specificity of targeting. In this study, we have tested the hypothesis of Hancock *et al.* (17) that the signal for mitochondrial importation resides within the 5′-flanking region and that 5′-extended precursor tRNAs are the native substrates for mitochondrial importation. We have shown that 5′- and 3′-end processing of tRNAs of all three classes occurs in the nucleus prior to export to the cytosol. These results imply that the substrates for importation into the mitochondrion are the mature tRNAs, at least in terms of end processing. The proposal that mature tRNAs are the import substrates *in vivo* is further supported by the finding that a T7-transcribed mature tRNA^{Ile} is imported into isolated mitochondria more efficiently than the 5′-extended precursor molecule.

The lack of editing at nucleotide 34 of the tRNA^{Trp} precursor molecules is consistent with a mitochondrial localization of this editing reaction and also with the importation of mature unedited tRNA^{Trp}. This observation is in contrast with the editing of tRNA^{Asp} in marsupials (38, 39) and mitochondrial tRNA editing in plants (40), where editing precedes tRNA 3′-processing. This difference might reflect the fact that in *L. tarentolae*, the edited tRNA is encoded in the nucleus and thus required for translation in both the cytosol and the mitochondria, whereas in plants and marsupials, the edited tRNAs are encoded in the mitochondria and only function in intra-organellar translation. In addition, tRNA editing in plants leads to stabilization of the acceptor stem and may be responsible for providing the proper recognition substrate for the 3′-processing activity.

The finding that edited *L. tarentolae* tRNA^{Trp} is poorly imported *in vitro* compared with unedited tRNA^{Trp} suggests a subtle molecular discrimination of isoacceptors by the transport apparatus. This discrimination of a single nucleotide change in the anticodon is reminiscent of the results of Rusconi

and Cech (2, 3) in *Tetrahymena*, in which a single nucleotide change in a $tRNA^{Gln}$ anticodon conferred a mitochondrial importation phenotype *in vivo*.

The presence of an intramitochondrial RNase P-like activity and a ATP/CTP:tRNA nucleotidyltransferase activity (16, 17) is not inconsistent with importation of mature tRNAs since the former activity could be involved in other aspects of RNA metabolism (41–43), and the latter activity could play a role in the repair of the 3′-ends of tRNAs. It should be noted, however, that our results with the mitochondrion-localized tRNAs we have examined do not eliminate the possibility that there are alternate pathways for importation of other tRNAs into the mitochondrion in these cells (19, 20). A complete cataloging of all mitochondrial tRNAs in the cell is required to address this question.

The data presented in this report suggest a model for a tRNA import pathway in *L. tarentolae*, which is diagrammed in Fig. 9. The tRNAs are transcribed in the nucleus as precursors with extended 5′- and 3′-ends, and the precursors are end-processed prior to export from the nucleus. The mature tRNAs are then exported to the cytosol, where a fraction is retained for cytosolic translation, and another fraction is imported into the mitochondrion for organellar translation.

Acknowledgments—We thank all members of the Simpson laboratory for discussion and assistance. We thank Dr. David Campbell and Gusti Zeiner for supplying the U6 snRNA sequence and the U6-1 oligonucleotide. We also thank Dr. Norman Pace for providing an *E. coli* RNase P RNA plasmid.

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