The mitochondrial tRNAs of Trypanosoma brucei are nuclear encoded and imported into the mitochondrion. A heterogeneous population of RNAs having characteristics of precursor tRNAs have previously been identified within the mitochondrion of T. brucei, suggesting that import occurs via a precursor molecule. In order to identify nuclear genes encoding tRNAs targeted to the mitochondrion, individual mitochondrial tRNAs were separated using two-dimensional gel electrophoresis and enzymatically sequenced. A 1.1-kilobase pair genomic DNA fragment was cloned containing three tRNA genes, tRNA<sub>Ser</sub>, tRNA<sub>Leu</sub>, and tRNA<sub>35294</sub>. Dicistronic precursors containing the tRNA<sub>Ser</sub> and tRNA<sub>Leu</sub> transcripts with a 59-nucleotide intergenic sequence were identified by reverse transcriptase and polymerase chain reactions and the 5′ end of the precursors determined. The dicistronic precursor tRNA is present both in the cytosol and the mitochondrion supporting a model for tRNA import involving precursor tRNA transcripts.

The mitochondrial import of a subset of nuclear encoded tRNAs has been reported in a number of different organisms including Tetrahymena (1, 2), Acanthamoeba (3), Paramecium (4), Chlamydomonas (5), Phaseolus (6), Solanum (7), and Triticum (8). Trypanosoma brucei (9) and Leishmania tarentolae (10) are two organisms in which all of the mitochondrial tRNAs are encoded in the nucleus.

The nuclear encoded tRNA genes in T. brucei have been categorized into three different classes: tRNAs specific to the cytoplasm, tRNAs specific to the mitochondria, and tRNAs that appear to be shared between both compartments (9). Sixteen tRNA genes have been identified in T. brucei (11–15). Two of these have been characterized as single copy genes and have been shown by hybridization studies to be present in both mitochondrial and cytoplasmic RNAs, suggesting that they are targeted to both compartments (14). The majority of the tRNA genes identified have been selected with a heterologous population of labeled tRNAs. Due to the ability of closely related tRNAs to cross-hybridize it is difficult to determine whether the other identified genes encode tRNAs targeted to the mitochondrion. The identification of nuclear genes encoding tRNAs targeted to the mitochondrion is crucial in identifying the substrate requirements and mechanism of mitochondrial RNA import.

Little is known about the mechanism by which a nuclear encoded RNA can be imported into the mitochondrion. It has been suggested that import of tRNAs may be in association with the cognate nuclear encoded aminoacyl tRNA synthetase of the tRNA (16). It has recently been shown that a tRNA<sub>Leu</sub> is imported into yeast mitochondria associated with the cognate synthetase (17). Transfection studies with both Trypanosoma and Leishmania have demonstrated mitochondrial import of endogenous, mutated, and exogenous tRNAs. These results suggest that the substrate requirement for import is contained within the mature tRNA structure, independent of the genomic 5′- and 3′-flanking sequences (18–20). An in vitro import system has been described in Leishmania in which import was demonstrated by a ribonuclease protection assay (21). An antisense transcript from the 5′ upstream region of β-tubulin appears to be imported, suggesting that import of small RNAs may be nonspecific in this system. More recent studies have suggested the presence of mitochondrial membrane proteins that serve as receptors for Leishmania tRNAs and the antisense tubulin transcript (22, 23). We have previously reported the presence of a population of potential precursor tRNAs within T. brucei mitochondria. These tRNAs were identified based on their ability to be posttranscriptionally labeled at their 3′ end by tRNA nucleotidyl transferase and the ability of RNase P to cleave them to the same size and pattern as mature tRNAs (24). This suggested that for at least a subset of tRNAs imported into the mitochondria of T. brucei, the import substrate consists of a precursor tRNA. Attempts to characterize these precursors by Northern blot hybridization and primer extension analysis have failed, suggesting that the initial identification of precursor molecules may have been artifactual or that the precursors are present in very low abundance (25).

In this report, we describe the cloning of a nuclear gene encoding a mitochondrial tRNA<sub>Leu</sub>. The tRNA<sub>Ser</sub> gene is immediately downstream of a tRNA<sub>Ser</sub> gene. This unusual genomic organization has been seen for other tRNA genes of T. brucei (13–15). Using a sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay a larger transcript containing tRNA<sub>Leu</sub> was identified within the mitochondrion. The 5′ end of this transcript was determined by 5′ RACE. This low abundance tRNA<sub>Leu</sub> precursor contains the tRNA<sub>Ser</sub>, a 59-nucleotide intergenic sequence and the tRNA<sub>Leu</sub>. This demonstrates the existence of an imported dicistronic precursor tRNA for a specific mitochondrial tRNA in T. brucei.

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† The abbreviations used are: RT, reverse transcriptase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; Pipes, 1,4-piperazinediethanesulfonic acid; AMV, avian myeloblastosis virus.

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**MATERIALS AND METHODS**

Trypanosoma brucei (TREU 667) was grown at 27 °C in a semi-defined medium (26) containing 10% heat-inactivated fetal bovine serum (Whittaker) and 20 μg/ml gentamicin sulfate. Mitochondria were isolated from cells at a density of 1–2 × 10^7 cells/ml as described previously (27). Briefly, cells were suspended in a hypo-osmotic buffer and then lysed by passage through a 26 gauge needle. The cytosolic RNAs were isolated from the supernatant after the mitochondria and membraneous material had been pelleted at 15,800 × g. Mitochondrial vesicles were isolated from a 20–35% Percoll gradient. Mitochondrial vesicles from 2 × 10^10 cells were solubilized in 300 μl of buffer containing 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8, 0.5% sodium dodecyl sulfate. The RNAs from both mitochondria and cytosol were extracted with hot phenol (1:1), and chloroform and then precipitated with sodium acetate and ethanol.

**Metabolic Labeling of Mitochondrial tRNAs**—Mitochondria isolated from 2.5 × 10^9 cells were resuspended in 5 mM Hepes, pH 7.6, 3 mM potassium phosphate, pH 7.7, 125 mM sucrose, 10 mM magnesium acetate, 1 mM EDTA, 0.5 mM mercaptoethanol, 0.5 mM ATP, 0.5 mM UTP, and 1 μCi of [α-^32P]CTP (NEN Life Science Products) in a volume of 1 ml. TCA in mitochondria are present in transcription due to a reduction in the mitochondrial GTP pool resulting from the preincubation in the absence of NTPs and the omission of GTP in the reactions. The mitochondria were incubated at room temperature for 30 min followed by centrifugation in a microcentrifuge for 30 s at room temperature. Mitochondria were solubilized and RNAs were isolated as described above. Isolated RNAs were loaded in a denaturing gel-loading buffer onto a 7% polyacrylamide/8 M urea gel. The labeled tRNAs were purified on an 8% polyacrylamide/8 M urea gel. The region of the gel containing the tRNAs was visualized by ethidium staining and excised. RNAs were either ethanol precipitated, and resuspended in 20 μl of distilled H2O. PCR reactions were performed in the absence of reverse transcriptase. After primer extension, the reactions were treated with 2 μl of 10 mg/ml RNase A for 10 min at 37 °C followed by incubation with 5 μl of 10 mg/ml proteinase K for 10 min at 37 °C. The reactions were phenol/chloroform extracted (1:1), ethanol precipitated, and resuspended in 20 μl of distilled H2O. PCR reactions were performed with the tRNA^leu^/cytochrome c-specific oligonucleotides. 2 μl of the primer extension reactions or 2 μl of a 1:20 dilution of the primer extension reactions were amplified using Taq Polymerase (Perkin-Elmer) for 30 cycles at an annealing temperature of 53 °C, an extension temperature of 72 °C, and a denaturing temperature of 92 °C. 20 μl of the 100-μl PCR reaction was electrophoresed in a 2.5% NuSieve agarose (FMC) gel. PCR bands were visualized by ethidium staining. Quantification of the PCR products was performed by the addition of 1.0 μl of [α-^32P]CTP (NEN Life Science Products) to PCR reactions. The NuSieve gel was dried down under vacuum in the absence of heat, and the radioactivity was quantitated using a Molecular Dynamics model 4000E PhosphorImager.

5′ RACE of Mitochondrial RNA—A 30-nucleotide synthesized RNA was ligated to mitochondrial RNA by combining 80 pmol of the synthetic RNA and 10 μg of mitochondrial RNA. The mixture was heated to 95 °C for 2 min, and the volume was then brought to 100 μl in a buffer containing a final concentration of 50 mM Hepes, pH 7.9, 20 mM MgCl2.
for 5 min. One-tenth of the ligation was then transformed into MAX T4 ligation buffer, T4 DNA ligase and incubated at room temperature. The reaction was added to 50 ng of TA vector, 1 μl ligase kit (Roche Molecular Biochemicals). Briefly, 1:100 of the PCR products were then ligated into the TA vector (Invitrogen) using the 5-min ligation reaction. 

The cDNAs were then extracted with phenol and phenol/chloroform (1:1) and precipitated with sodium acetate and ethanol. For reverse transcription, 2 μg of the ligation reaction was added to oligonucleotide 20, incubated at 85 °C for 5 min, and placed on ice. Then a mixture containing 500 μM of each deoxynucleotide, 1× Enhanced AMV buffer (Sigma), 1 unit/μl Enhanced AMV-RT (Sigma), and 1 unit/μl RNasin (Sigma) was added. The reactions were incubated at 55 °C for 1 h and then 95 °C for 5 min. A PCR reaction containing 5 μl of the RT reaction, 200 μM deoxynucleotides, 1× PCR buffer (Perkin-Elmer), 1 pmol 5’ oligonucleotide 002, and 1 pmol 3’ oligonucleotide 7 was amplified using Taq Polymerase (Perkin-Elmer) for 35 cycles at a denaturing temperature of 94 °C, an annealing temperature of 55 °C, and an extension temperature of 72 °C. The PCR amplified cDNAs were collected by phenol/chloroform (1:1) extraction and ethanol precipitated with sodium acetate. The cDNAs were then ligated into the TA vector (Invitrogen) using the 5-min ligation kit (Roche Molecular Biochemicals). Briefly, 1:100 of the PCR reaction was added to 50 ng of TA vector, 1× DNA dilution buffer, 1× T4 ligation buffer, T4 DNA ligase and incubated at room temperature for 5 min. One-tenth of the ligation was then transformed into MAX efficiency DH5α competent cells (Life Technologies, Inc.), and the 5’ ends of the resulting clones were determined by dideoxynucleotide sequencing using the 3’ oligonucleotide 7.

Nucleotide Sequence Accession Number—The sequence identified in this paper has been assigned GenBank™ accession number U63718.

RESULTS

Identification of Precursor tRNAs within the Mitochondria of T. brucei—In vitro labeling of a population of potential precursor tRNAs was accomplished as in previous studies (24). Isolated mitochondria were incubated in the presence of [α-32P]CTP in the absence of transcription. RNAs were isolated from these organelles and separated on a 7% polyacrylamide/4 M urea gel. The RNAs were visualized either by ethidium staining or by autoradiography. B, the entire lane was excised and separated on a 17.6% polyacrylamide/8 M urea gel and visualized by autoradiography.

The RNAs were visualized either by ethidium staining or by autoradiography (Fig. 1). As a control, a precursor tRNA Asp from these organelles was ligated circular tRNAs that are substrates for processing by RNase P from E. coli, and Fig. 2). The RNAs that are substrates for processing by RNase P from E. coli are likely to be tRNA with long 5’ termini. However, a significant portion of the RNA population, containing circular tRNAs and putative precursor tRNAs, are not substrates for processing by RNase P and Fig. 2). The RNAs that are substrates for processing by RNase P are likely to be tRNA with long 5’ extensions.

Identification of Nuclear Genes Encoding Mitochondrial tRNAs—A number of nuclear encoded tRNA genes have been identified in T. brucei. Identification of these genes has been based on analysis of cloned genomic fragments (14) or by screening with a heterogeneous population of labeled tRNAs (11–15). In order to identify genes specifically encoding mitochondrial tRNAs of T. brucei, we have isolated and sequenced...
individual mitochondrial tRNAs. RNAs were isolated from purified preparations of *T. brucei* mitochondria (27). RNAs were also prepared from a cytosolic fraction following sedimentation of organelles at 12,000 × g. The cross-contamination of the RNAs from the cytosol and mitochondria was established by hybridization with probes specific for mitochondrial and cytosolic RNAs (see Fig. 6, C and D). In order to isolate individual tRNAs, mitochondrial and cytosolic RNAs were separated on a 7% polyacrylamide/4 M urea gel (Fig. 3A). Gel slices corresponding in size to the mature tRNAs indicated by the bracket in Fig. 3A were excised and separated on a 17.6% polyacrylamide/8 M urea gel. The tRNAs were visualized by ethidium staining (Fig. 3B). Comparison of the pattern of tRNAs from the mitochondria versus the cytoplasm shows several differences. Most of the tRNAs appear to be shared between the two compartments (including RNAs 1, 3 and 4 indicated by *small* arrows), but several are compartment specific (*large* arrows).

Fifteen individual tRNAs were excised from the two-dimensional gel and eluted. The tRNAs were then 5′ or 3′ end-labeled and subjected to enzymatic sequencing (data not shown). Clear sequence was obtained from all of these RNA indicating the purity of the products from the two-dimensional gel purification. The sequence obtained from one 3′ end-labeled tRNA (arrow 1 in Fig. 3D) was used to design a complementary oligonucleotide designated D1.

The specificity of oligonucleotide D1 for a single tRNA was confirmed by Northern blot hybridization of two-dimensional gels of mitochondrial and cytosolic tRNAs (Fig. 4). Oligonucleotide D1 hybridizes to the single tRNA, from which its sequence was derived, in both the cytoplasm and the mitochondrion. The second tRNA that appears to hybridize in the cytosolic tRNAs is a degradation product that occurred during the two-dimensional separation. Enzymatic sequencing of this tRNA species was identical to tRNA 1 except for the absence of a single nucleotide at the 3′ terminus (data not shown).

Oligonucleotide D1 was used to screen a genomic library derived from *T. brucei*. A 1.1-kilobase pair genomic fragment was isolated that contained sequence corresponding to the tRNA hybridizing to oligonucleotide D1. The tRNA was identified based on its anticodon as a tRNALeu. This genomic fragment also contains two closely related tRNA3ser genes that are shown diagrammatically in Fig. 5. The tRNA3ser and tRNA1aur are oriented in the same direction and are separated by 59 nucleotides. The tRNA3ser is in the opposite orientation and is separated from tRNA1aur by 210 nucleotides. The sequence and secondary structure predictions for these tRNAs are shown in Fig. 5.

To confirm that the genomic sequence corresponds to the original enzymatically sequenced tRNA, a Northern blot of a two-dimensional gel of mitochondrial and cytosolic tRNAs was hybridized with a labeled PCR product containing the genomic tRNA1aur sequences (data not shown). High stringency washes identify the single tRNA encoded by the genomic sequence, corresponding to the original tRNA that was enzymatically sequenced. tRNA3ser and tRNA1aur hybridize to tRNAs identified as arrows 3 and 4, respectively in Fig. 3B in both the mitochondrion and cytosol.2 These results indicate that the 1.1-kilobase pair genomic fragment encodes three tRNAs that are targeted to both the mitochondrion and the cytoplasm.

**tRNA1aur Precursor Identification**—In order to determine whether a precursor tRNA1aur was present in the mitochondrion and cytoplasm, we developed a sensitive RT-PCR method. The tRNA1aur D1 oligonucleotide complementary to the variable loop and T-stem region of mature tRNA1aur was annealed to mitochondrial or cytosolic RNAs and was extended using reverse transcriptase. The single-stranded DNA products were amplified with the D1 oligonucleotide and each of 4 oligonucleotides complementary to sequences upstream of the mature tRNA1aur genomic sequence. The position of the oligonucleotides used and the expected size products are shown in Fig. 6A. Control reactions were performed with both mitochondrial and cytosolic RNAs in the absence of reverse transcriptase to rule out the possibility of DNA contamination. Additional control reactions with DNA as a target or in the absence of target DNA show the predicted size products of the PCR reactions and eliminate the possibility of contamination of the solutions used in the PCR reactions. The identities of the predicted size D1, 1–4 and D1, 1–15 products from the mito-

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chondria and cytosolic reactions as well as the predicted size D1, 1–14 product in the cytosolic reaction were confirmed using nested PCR (data not shown). Additional bands in the cytosolic D1, 1–15, D1, 1–14, and D1, 1–13 reactions have not been identified. The RT-PCR results show that a precursor tRNA is present both in the cytoplasm and the mitochondrion, extending a minimum of 60 nucleotides upstream of the mature 5' end of tRNA\textsubscript{\text{La}} (Fig. 6B). Furthermore a precursor extending over 50 nucleotides upstream of the mature tRNA 5' end can be identified within the cytoplasm as shown by the oligonucleotides D1, 1–15 product found in the cytosolic RNA. This indicates that the cytoplasmic precursor extends through the intergenic region.

The possibility that the precursor tRNA identified within the mitochondria by RT-PCR was due to cytosolic contamination of the mitochondrial RNA preparation was addressed by two ap-
Mitochondrial tRNA Import

proaches. Mitochondrial and cytosolic RNAs were separated on a polyacrylamide gel, Northern blotted, and probed with cytosolic and mitochondrial-specific probes (Fig. 6C). An oligonucleotide complementary to the 140-nucleotide spliced leader RNA of *T. brucei* hybridized preferentially to the cytosolic RNAs. An oligonucleotide complementary to the mitochondria enriched tRNA 5, identified by typical migration on two-dimensional gels (Fig. 3B) and analyzed by partial enzymatic sequencing (data not shown), preferentially hybridized to the mitochondrial tRNAs. Quantitation of the hybridization to these RNAs suggests that cytosolic contamination of the mitochondrial RNA was approximately 1%. Less than 5% of the cytosolic tRNA pool was a consequence of leakage of mitochondrial RNAs during mitochondrial purification.

In addition, a control primer extension reaction was performed using an oligonucleotide complementary to cytosolic cytochrome c mRNA. PCR reactions were carried out under the same conditions used for the RT-PCR of the precursor tRNAs (Fig. 6D) except that the amount of RT product used in these reactions was reduced 200-fold. Quantitation of the PCR product corresponding to the cytochrome c mRNA shows that the cytosolic contamination of the mitochondrial RNA preparation is approximately 0.4%. This demonstrates that the precursor tRNA^{Leu} is present in the mitochondrion at a 25–60-fold higher amount than can be explained by simple cytosolic contamination of the mitochondrial RNAs.

The RT-PCR products seen in Fig. 6B were difficult to interpret because it was not possible to determine whether the lack of a PCR product indicated the 5' end of the precursor or a truncated product resulting from the inability of the reverse transcriptase to extend through the upstream tRNA. In addition, it is possible that multiple 5' ends exist within the mitochondrial RNA pool. These possibilities were addressed by 5' RACE of total mitochondrial RNA (Fig. 7). Total *T. brucei* mitochondrial RNA was ligated to a 30-nucleotide synthetic RNA and reverse transcribed with the oligonucleotide 20, which is complementary to the intergenic region immediately upstream of the tRNA^{Leu}. To decrease premature termination because of RNA secondary structure, reactions were done at 55 °C using the Enhanced AMV-RT (Sigma). The cDNAs were then amplified with oligonucleotide 002, complementary to the ligated oligonucleotide, and oligonucleotide 7, complementary to the tRNA^{Ser}/tRNA^{Leu} intergenic sequence. PCR products were cloned into the TA vector and sequenced using oligonucleotide 7. Three independent clones containing intergenic sequence were identified. The sequence of each clone showed that the synthetic RNA was ligated to a dicistronic precursor 14 nucleotides upstream of the mature 5' end of the tRNA^{Ser}. These results are consistent with the precursor transcript identified in these studies being a dicistronic tRNA^{Ser}/tRNA^{Leu} formed by polymerase III transcription read-through of the upstream tRNA^{Ser}.

**DISCUSSION**

We have previously defined a population of mitochondrial RNAs with characteristics of precursor tRNAs (22). A subset of the precursor size molecules can be processed by an *E. coli* RNase P RNA to mature mitochondrial tRNAs. These findings suggest that import of at least a subset of the tRNAs may require sequences flanking the tRNAs. In this paper we have identified the nuclear genes encoding two tRNAs targeted to the mitochondrion and determined that a dicistronic precursor tRNA exists within the mitochondrion.

We have separated individual mitochondrial and cytosolic tRNAs using two-dimensional gel electrophoresis (Fig. 3B). A complementary oligonucleotide was designed based on the partial enzymatic sequence of one mitochondrial tRNA. This oligonucleotide was used to identify a genomic DNA fragment containing three tRNAs, tRNA^{Ser}_{5}, tRNA^{Leu}_{5}, and tRNA^{Ser}_{2} (Fig. 5).

The genomic organization of the tRNA genes of *T. brucei* is unusual (11–15). tRNA^{Ser}_{5} and tRNA^{Leu}_{5} are closely linked, separated by only 59 nucleotides. This genomic arrangement may play a role in distinguishing between tRNAs targeted to the mitochondrion or targeted to the cytoplasm. We have shown that the tRNA^{Ser}_{5} and tRNA^{Leu}_{5} genes are transcribed to give a dicistronic precursor transcript. Normally RNA polymerase III initiates tRNA transcription 10–20 nucleotides upstream of the mature 5' tRNA sequence (reviewed in (29)). The 5' terminus of the dicistronic precursor tRNA was determined to be 14 nucleotides upstream of the tRNA^{Ser}_{5}. This suggests that the precursor tRNA^{Ser}_{5}/tRNA^{Leu}_{5} is formed by the inability of polymerase III to efficiently terminate the transcription of the upstream tRNA^{Ser}_{5}. The presence of the tRNA^{Ser}_{5}/tRNA^{Leu}_{5} dicistronic molecule within the mitochondrion implies that these precursor tRNAs must somehow escape the tRNA processing events that normally occur within the nucleus. A tRNA precursor consisting of two linked tRNAs may fold into an unusual structure (30), which could allow the precursor to escape nuclear processing. Alternatively, the precursor molecule may be recognized by specific RNA binding proteins that participate in the export of the unprocessed RNA from the nucleus and its subsequent import into the mitochondrion. In mammalian cells it has recently been demonstrated that processing of precursor tRNAs by RNase P is modulated by the La antigen phosphoprotein (31). A similar pathway for protein-mediated protection

**FIG. 7.** Identification of the 5' end of the precursor tRNA^{Leu} transcript in mitochondria. A 30-nucleotide synthesized RNA (hatched box) was ligated to mitochondrial RNA. The ligated RNA was then reverse transcribed with oligonucleotide 20. The resulting cDNAs were then PCR amplified using both the 5' oligonucleotide 002, which is complementary to the 30-nucleotide ligated RNA, and the nested 3' oligonucleotide 7. The products from the PCR reaction were ligated into the TA vector and sequenced using oligonucleotide 7. The 5' end of the precursor tRNA^{Leu} transcript was mapped to be 14 nucleotides upstream (closed triangle) of the predicted mature 5' end of the tRNA^{Ser} (open triangle).
of precursor tRNA1Ser may allow it to be exported from the nucleus in an unprocessed form.

Some processing events may occur prior to, during, or shortly after mitochondrial import. Processing or modifications of the precursor tRNA1Ser/tRNA1Leu once inside the mitochondria may allow these transcripts to be recognized and processed into mature functional tRNAs by the mitochondrial RNase P and nucleotidyl transferase (24). In support of this, a mitochondrial specific modification at position 32 of tRNA1Leu was identified as 2′-O-methylcytidine and occurs following mitochondrial tRNA import (20).2 Import of tRNAs does not appear to be dependent on this modification, and the importance of this modification in forming functional mitochondrial tRNAs is not known (20). We are currently investigating modifications of imported tRNAs and the roles these modifications may play in correctly processing precursor tRNAs and forming functional mature tRNAs.

In previous studies (22), oligonucleotides complementary to conserved sequences in tRNAs (D-loop and stem) were used in both Northern hybridization and primer extension reactions to identify putative precursor tRNAs. When similar experiments were performed using an oligonucleotide complementary to specific sequences immediately upstream of the mature tRNA1Leu 5′ terminus, no products were observed in the mitochondrial or the cytosolic fractions.2 This is consistent with a recent study indicating that precursor molecules with 5′ extended sequences were not detected either by primer extension analysis or Northern blot hybridization (25). In this report we have used a highly sensitive RT-PCR assay to identify precursor tRNA1Leu molecules within the mitochondrion and cytosol, suggesting that precursor tRNA1Ser/tRNA1Leu is present at very low abundance in T. brucei. We now believe that the majority of higher molecular weight RNAs observed to incorporate radiolabeled CTP in a metabolic labeling experiment (Fig. 1A) are actually artificial ligation products that are formed during our mitochondrial isolation procedure. We have observed during metabolic labeling experiments with isolated mitochondria that over time there is an increase in both CTP incorporation and intensity of ethidium staining in precursor size RNA molecules with a corresponding decrease in mature tRNA molecules.2 This would explain why only a small percentage of the metabolically labeled precursor tRNAs are cleaved in our RNase P assay (Ref 24 and Fig. 2).

Transfection studies have been used to investigate the determinants of tRNA import into trypanosome mitochondria (18, 19, 21). The results from these studies suggest that the mature tRNA structure alone can directly import several tRNAs into the mitochondria, irrespective of their genomic context or genetic origin. These findings are an apparent contradiction to our findings. Although we do not have an explanation for these differences, it is possible that the high levels of tRNAs expressed from the episomal vectors used in the transfection studies may have influenced the distribution of tRNAs. The studies reported here do not directly address whether the dicistronic precursor or mature tRNAs are preferentially imported into trypanosome mitochondria. However, the data demonstrate the presence of a nuclear encoded, dicistronic, tRNA1Ser/tRNA1Leu precursor within the mitochondria. Recently, we have investigated the import of tRNAs into the mitochondria of T. brucei. The dicistronic tRNA1Ser/tRNA1Leu molecules are efficiently imported in this in vitro system, whereas mature tRNA1Ser and tRNA1Leu are poor import substrates.3

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