A Sequence Motif within Trypanosome Precursor tRNAs Influences Abundance and Mitochondrial Localization

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Trypanosoma brucei lacks mitochondrial genes encoding tRNAs and must import nuclearly encoded tRNAs from the cytosol. The mechanism and specificity of this process remain unclear. We have identified a unique sequence motif, YGG(C/A)RRC, upstream of the genes encoding mitochondrially localized tRNAs in T. brucei. Both in vitro import studies and in vivo transfection studies indicate that deletion of the YGG(C/A)RRC sequence alters mitochondrial localization of tRNA^Leu, and in vivo studies also show a decrease in the cellular abundance of tRNA^Leu. These studies provide direct evidence for cis-acting RNA motifs within precursor tRNAs that facilitate the selection of tRNAs for mitochondrial import in trypanosomes. Furthermore, we found that mutations to the YGG(C/A)RRC sequence also altered the intracellular distribution of other endogenous tRNAs, suggesting a general role for this sequence in tRNA trafficking in trypanosomes.

Mitochondria typically encode all of the rRNAs and tRNAs necessary for autonomous protein synthesis. However, import of nuclearly encoded tRNAs has been documented in evolutionarily diverse species, including yeast, protozoa, plants (reviewed in reference 25), and marsupials (6). The numbers and identities of imported tRNAs are highly variable, but the only organisms identified to date that must import all of their mitochondrial tRNAs are the kinetoplastid protozoa Trypanosoma and Leishmania (7, 8, 28).

tRNAs contain highly conserved intragenic RNA polymerase III promoter elements and a nearly ubiquitous tertiary structure (7). The sequence and structure conservation of tRNAs complicates how they might be specifically targeted to mitochondria. Most of the tRNAs in kinetoplastid genomes are shared between the cytosol and mitochondrion but are localized to the mitochondrion with differing efficiencies, ranging from 1 to 7.5% of the total cellular tRNA in Trypanosoma brucei (30). tRNA structure is important for mitochondrial localization in T. brucei; however, this alone may not explain the differences in the abundance of imported tRNAs (11). It has been proposed that mitochondrially localized tRNAs in Leishmania may contain sequences that positively influence targeting and transport into the mitochondrion (3, 4, 16, 18, 22). A positive import determinant was discovered in the D arms of Leishmania tRNA^Leu (16) and tRNA^Tyr (18). Swapping the D arms of imported tRNA^Leu and a cytosolically localized tRNA^Glu conferred mitochondrial import to the hybrid tRNA^Glu, but did not eliminate mitochondrial localization of the hybrid tRNA^Leu, suggesting that there are multiple signals for import (16). In vitro studies with Leishmania mitochondria suggested that there might be different sequence or structural requirements for crossing the outer and inner membranes of mitochondria, indicating that import may involve two distinct steps (3). Furthermore, a SELEX (systematic evolution of ligands by exponential enrichment) procedure was used to isolate sequence aptamers that were imported into mitochondria with high efficiencies. One set of the import-competent aptamers contained the motif YGGYAGAGY, which is present in the anticodon or D arms of many tRNAs, whereas another set contained the motif UG_3_U, found in the V-T region of other tRNAs. These aptamers were able to interact with the inner membranes of isolated Leishmania mitochondria. Interestingly, the first motif is found in the D arm of the imported tRNA^Tyr and the second is found in the imported tRNA^Leu (4). Although the previous studies suggest the presence of positive import determinants, an alternative view proposes that the mitochondrial import machinery may not discriminate between different tRNAs but may be negatively regulated by sequences or nucleotide modifications that inhibit import (12).

Although the role of 5′ flanking sequences in localization of tRNAs to the mitochondria of trypanosomes is highly debated, previous studies have shown the presence of tRNA precursors in the mitochondria of T. brucei (15). Transcription of one precursor tRNA was shown to initiate 14 nucleotides upstream of the tRNA^Ser coding sequence and extend through a 59-nucleotide intergenic sequence and a downstream tRNA^Leu (15). These findings suggest that at least some tRNAs may be imported as precursors rather than as processed, mature tRNAs (15). Consistent with this result is the presence of RNase P activity in T. brucei mitochondria, although the ability of this activity to process in vitro imported precursor tRNAs to a mature size has not been demonstrated (9, 23).

In this paper, we present evidence that sequences upstream of tRNA coding regions, within the 5′ leader sequence, influence localization of tRNAs in T. brucei. Sequence analysis of the immediate 5′ flanking sequences of T. brucei tRNAs revealed the presence of a highly conserved dinucleotide GG within a conserved sequence motif, YGG(C/A)RRC. By 5′ rapid amplification of cDNA ends (RACE), we have determined that precursor tRNA^Leu, including this motif within the 14-nucleotide 5′ leader, is localized in both the cytosol and the...
mitochondrion. Interestingly, this sequence is similar to the previously published motif YGGYAGGY found in import-competent tRNAs and aptamers in Leishmania (3, 4). Using an in vitro import system, we tested 5’ deletions of a tRNA precursor for the ability to be imported into mitochondrion. A significant decrease in import was observed when the YGG(C/ A)RRC motif was removed from the precursor tRNALeu. We also developed an in vivo system to further characterize the influence of flanking sequences on import. Mutations to the YGG(C/A)RRC sequence indicate that this 5’ flanking sequence is involved in maintaining both the abundance and cellular distribution of tRNALeu. Finally, not only does mutation of this sequence motif affect the localization of the tagged tRNALeu, it also has a global effect on localization of other endogenous tRNAs to the mitochondrion.

MATERIALS AND METHODS

Trypanosome growth, purification of mitochondria, and isolation of RNA. *T. brucei* procyclic cells (TREU 667) were grown at 27°C in semidefined medium (5) containing 10% heat-inactivated fetal bovine serum (Sigma) and 20 μg of gentamicin sulfate (Life Technologies, Inc.) per ml. Mitochondria were isolated from cells at a density of 1 × 10^6 to 1.5 × 10^7 cells/ml as described previously for in vitro import assays (21, 32). Mitochondria were isolated from 4 to 8 liters of culture at a cell density of 1 × 10^7 to 1.5 × 10^7 cells/ml as described previously for in vivo import studies (10). Briefly, cells were suspended in hypotonic buffer and then lysed by passage through a 26-gauge needle. Mitochondrial vessels were isolated from a 20 to 35% Percoll gradient. Mitochondrial vessels were treated with 0.6 U of micrococcal nuclease (USB Corporation) per 3.5 × 10^6 cells in 1 ml of 10% glycerol–10 mM Tris-HCl (pH 8.0)–1 mM CaCl2 for 20 min at room temperature. The reaction was stopped by the addition of 0.5 M EDTA, pH 8.0, to a final concentration of 10 mM. The vessels were recovered by centrifugation at 32,500 × g for 15 min (adapted from reference 8). The mitochondrial RNA was extracted from the vessels with TriPure isolation reagent (Boehringer Mannheim) according to the manufacturer’s instructions. Cytosolic RNA was isolated from procyclic *T. brucei* (TREU 667) as described previously (8), except that the RNA was extracted with TriPure isolation reagent (Boehringer Mannheim) after treatment with DNase I (Roche).

In vitro import of 5’ deleted tRNAs. The 5’ deletions of the dicistronic tRNA*Fl (25)–tRNA*Leu(CAA) (25) were constructed from the previously described D1 genomic clone for in vitro import (15). The 5’ oligonucleotides for PCR amplification were generated by mixing 2 μM primer 19 (5’-GTTTTGTCAGGTGCAATATG-3’) and primer 4 (5’-GTTGTCCTAACACAACTGGTTTGG-3’); primer 5 (5’-GGCAAGTGTCGGAGTGTCC-3’), or primer 1 (5’-GTTGGGGGGCGGTGTCACC-3’). The PCR conditions were as follows: denaturing at 94°C for 4 min, annealing at 50°C for 2 min, extension at 68°C for 6 min, continued with 10 cycles of 94°C for 1 min, 56°C for 2 min, and 68°C for 5 min, and finished with a final cycle of 68°C for 8 min. PCR products were digested with *Kpn* and *Cla* to allow targeted integration in a nontranscribed region of the dicistronic precursor tRNA*. The resulting mutated fragment was digested with *Kpn* and *Cla* and cloned into the *Kpn* and *Cla* sites of vector pXS2-Cl. The resulting vector was pSer-Leu*1. All of the following constructs were created with the ExSite PCR-based site-directed mutagenesis kit (Stratagene). The kit was used according to the manufacturer’s instructions. Cytosolic RNA was isolated from procyclic *T. brucei* (TREU 667) as described previously (8), except that the RNA was extracted with TriPure isolation reagent (Boehringer Mannheim) after treatment with DNase I (Roche).

Transfection of trypanosomes. The following protocol was adapted from the work of Bangs et al. (2). All vectors were Qiagen (Qiagen, Inc.) purified and linearized with *Cla* to allow targeted integration in a nontranscribed region of the mitochondrial tRNA*Leu* of the tRNA*Ser–Leu* gene cluster (see Fig. 3A). The 5’ flanking sequence for all integrated vectors was retained as the 5’ flanking sequence of the endogenous tRNA*Leu* because we used this site for integration. Each vector was phenol extracted once, chloroform extracted four times, precipitated with 100% ethanol, washed with 70% ethanol, and resuspended to 100 ng/ml in OptiMEM medium (Life Technologies, Inc.) Mid-log-phase (5–10^6 cells/ml) procyclic trypanosomes were washed once in ice-cold phosphate-buffered saline and resuspended at 4 × 10^7/ml in OptiMEM medium (Life Technologies, Inc.). OptiMEM (0.1 ml) containing linearized vector (total of 1 μg) was added to 0.5 ml of cells (2 × 10^5) in a 0.4-mm-wide cuvette (Bio-Rad) on ice. The cuvette was electrooporated (1.4 kV, 25 μF with two pulses, 10 s apart, in a Bio-Rad gene pulser. Cells were transferred to 10 ml of prewarmed (27°C) semimedium, and at 48 h G418 (Invitrogen) was added to 50 μg/ml. Stable cell lines typically grew out in 10 to 14 days. Cultures were maintained with 25 μg of G418 per ml.

Primer extension analysis of tRNA localization in vivo. Each of the following oligonucleotides (Invitrogen) was labeled for use in primer extension reactions. Oligonucleotide 98S (5’-TATTGCGATACCCATTTACG-3’) is complementary to nucleotides 346 to 364 of the mitochondrial 25S rRNA (29). Oligonucleotide SL2 (5’-GTACAGAACATCTTTATTTATAG-3’) is complementary to the cytosolic 39-nucleotide spliced leader RNA (19). Oligonucleotide LE4 (5’-GTTGGAATCCGCCTTTATTTATTGAG-3’) is complementary to nucleotides 48 to 75 of tRNA*Leu*. Oligonucleotide CL (5’-GTTGGAATCCGCCTTTATTTATTGAG-3’) is complementary to nucleotides 46 to 67 of tRNA*Met* (3A). Oligonucleotide Met i (5’-CCGGTTTTTTGTTCTTTACGGC-3’) is complementary to nucleotides 47 to 67 of tRNA*Met* (3A). Each oligonucleotide was 5’ labeled with T4 polynucleotide kinase (New England Biolabs) under the following conditions: 100 pmol of oligonucleotide, 1× T4 polynucleotide kinase buffer, 20 μl of polynucleotide kinase, and 300 μCi of 32P-ATP at 1 h at 37°C. The oligonucleotide was subsequently annealed on a 10% polyacrylamide–8 M urea gel. The oligonucleotides were excised from the gel and eluted overnight in 0.5 M ammonium acetate–1 mM EDTA, pH 8.0, at room temperature. After pre-
cipation with 100% ethanol and washing with 70% ethanol, the oligonucleotides were resuspended in double-distilled H₂O at 2.25 pmol/µl.

The level of purity of the mitochondrial and cytosolic RNAs was tested by a primer extension assay. Both the mitochondrial and cytosolic RNAs extracted from the different transfectant lines were assayed for 9S rRNA and spliced leader RNA with oligonucleotides 95b and SL2, respectively. Each primer extension reaction contained the following: 1 µl of primer (2.25 pmol), 4 µl of 5 X avian myeloblastosis virus reverse transcriptase (RT) buffer (Promega), 1 µl of 5 mM dUTP (250 µM final concentration), 1 µl of 10 mM dATP, dTTP, and dCTP mix (500 µM final concentration), 1 µl of RNAin (Promega), 1 µg of RNA, and double-distilled H₂O to 18 µl. The primer extension reactions were performed in an MJ Research thermal cycler. The reactions were denatured at 94°C for 3 min and annealed at 55°C for 30 min. Two microliters of avian myeloblastosis virus RNA virus RT (20 U) (Promega) was added to each reaction, and extension was performed at 48°C for 35 min, followed by a 5-min denaturation step at 85°C. The reactions were analyzed by running on a 10% polyacrylamide-8 M urea gel. Quantitation was performed with a Molecular Dynamics Phosphorimager (model STORM-880). Mitochondrial and cytosolic preparations were used for subsequent analysis if they yielded contamination levels under 3%.

All of the transfectant lines, pSer-Leu*, pSer-Leu* 1, pSer-Leu* 2, pLeu* (+), and pLeu* (−), were assayed for the presence of tagged RNAe and endogenous tRNALeu in both cytosolic and mitochondrial fractions with oligonucleotide LE4, using the assay conditions listed above. Oligonucleotide Met e was used to assay for the presence of RNAmet in mitochondrial and cytosolic fractions for all of the transfectant lines under the same assay conditions. Oligonucleotide Met e1 was used to assay for RNAmet e1 in both RNA fractions for all of the transfectant lines. The assay conditions for oligonucleotide Met e required substitution for dCTP for ddCTP and dATP, dTTP, and dGTP mix for the ddATP, dTTP, and ddCTP mix listed above. The reactions were analyzed by running on a 10% polyacrylamide-8 M urea gel. Quantitation was performed with a Molecular Dynamics Phosphorimager (model STORM-880). The data were analyzed by calculating the ratio of mitochondrial to cytosolic RNA. This number was normalized to 1 for the results of the tagged transfectant line, pSer-Leu*, and all of the other transfectant lines were compared to that number. An import level of 1 indicates the endogenous steady-state level of each tRNA.

5’ RACE of mitochondrial and cytosolic RNA. The following protocol was adapted from that of LeBlanc et al. (15). A 27-nt RNA oligonucleotide (5’-CG UACCCGGCAGUAUG CUGAGGUAUAC-3’) (DHARMACON) (500 pmol) was ligated to 10 µg of mitochondrial and cytosolic RNA from pSer-Leu* and pSer-Leu* 1 transfectants. The mixture was heated to 95°C for 2 min, and the volume was adjusted to 100 µl with final concentrations of 50 mM Tris-HCl (pH 7.8), 20 mM MgCl₂, 10 mM dithiothreitol, 10 µM of bovine serum albumin per ml, 10 mM ATP, 10% dimethyl sulfoxide, and 0.4 U of RNasin per µl. Twenty units of T4 RNA ligase was added and the reactions were incubated at 16°C for 2 h. The RNAs were extracted with phenol and phenol-CHCl₃ (1:1) and precipitated with 100% ethanol and washing with 70% ethanol, the oligonucleotide LE4, using the assay conditions listed above. Oligonucleotide Met e was used to assay for the presence of RNAmet e in both RNA fractions for all of the transfectant lines. The assay conditions for oligonucleotide Met e required substitution for dCTP for ddCTP and dATP, dTTP, and dGTP mix for the ddATP, dTTP, and ddCTP mix listed above. The reactions were analyzed by running on a 10% polyacrylamide-8 M urea gel. Quantitation was performed with a Molecular Dynamics Phosphorimager (model STORM-880). The data were analyzed by calculating the ratio of mitochondrial to cytosolic RNA. This number was normalized to 1 for the results of the tagged transfectant line, pSer-Leu*, and all of the other transfectant lines were compared to that number. An import level of 1 indicates the endogenous steady-state level of each tRNA.

RESULTS

Presence of a novel sequence motif upstream of T. brucei tRNAs. In order to identify sequence elements in mitochondrially localized tRNAs in trypanosomes, we examined 5’ flanking and intragenic sequences of both mitochondrially localized tRNAs and the cytosolically localized tRNAMet e for conserved motifs. Although 50 putative tRNA genes have been identified in the trypanosome genome, we selected 23 tRNAs for which both expression and cellular localization have been determined (30). Similar analyses were completed with genes for tRNAs from yeast and humans.

The start site for RNA polymerase III transcription of the tRNAe and tRNA Ser genes is 14 nucleotides upstream of the 5’ ends of the mature tRNAs (see Fig. 2C) (15). This led us to examine immediate 5’ flanking sequence as potential leader sequences for the other tRNA genes. Alignments were done with 14 nucleotides of upstream sequence plus tRNA coding sequence through position 37 for tRNAs from T. brucei, Saccharomyces cerevisiae, and humans (Fig. 1). As expected, tRNA genes from all three organisms have highly conserved sequences within the tRNA coding sequence, including the A box promoter element, which overlaps with the D arm. Sequences upstream of the human and yeast tRNAs showed low degrees of sequence conservation, with the exception of a highly conserved A at position −10 in yeast. Alignments of T. brucei tRNAs revealed a highly conserved dinucleotide GG within a consensus sequence, YGG(C/A)RRC, located within 12 nucleotides upstream of the tRNA coding sequences. The dinucleotide GG has a substitution of one of the G residues in 4 of the 23 tRNAs analyzed, but the residue remains a purine in each of these cases. Remarkably, this upstream flanking sequence is similar to a sequence (YGGYAGAGC) identified as an import determinant in the D arm of mitochondrial localized tRNAs in Leishmania (3, 4, 16, 18). Furthermore, the sequence found in the D arm in Leishmania, YGGYAGAGC, overlaps with the conserved A box sequence found in the D arms of all tRNAs, including those in humans and yeast, whereas the motif we have identified upstream of trypanosome tRNAs is not present upstream of human or yeast tRNA genes.

Deletion of the YGG(C/A)RRC motif causes a decrease in import efficiency in vitro. Previous work showed the presence of a dicistronic precursor tRNA in the T. brucei mitochondrion (15). Furthermore, we have shown that this precursor RNA is efficiently imported in vitro by an ATP- and proton gradient-dependent pathway (32). In order to evaluate the role of 5’ leader sequences, including the YGG(C/A)RRC motif, in import, we prepared 5’ deletions of the dicistronic tRNA Ser, tRNA Leu substrate. Figure 2A shows a schematic diagram of this RNA import substrate, including the positions of the YGG(C/A)RRC motifs. The 241-nucleotide substrate begins at the start site for tRNA Ser transcription and consists of a 14-nucleotide 5’ leader sequence, tRNA Ser, a 59-nucleotide intergenic region, downstream tRNA Leu, and a 3’-terminal CCA. The start site for transcription of the tRNA Ser was determined by 5’ RACE (15). Progressive deletions of this substrate were constructed (Fig. 2A).

Substrate binding to (Fig. 2B, lanes B) and import into (Fig. 2B, lanes I) mitochondria were determined for each of the RNAs described above. Binding represents both RNA bound to the mitochondria and imported RNA. Importation of RNA is defined as RNA that is resistant to digestion by micrococcal nuclease treatment of the isolated mitochondrion. To control for the possibility of nonspecific resistance to micrococcal nuclease, mitochondria were lysed with detergent prior to micrococcal nuclease treatment (Fig. 2B, lanes C). Consistent with our previous results, the dicistronic tRNA Ser−tRNA Leu is imported efficiently into the isolated mitochondrion (Fig. 2B) (32). Deletion of tRNA Ser and its 5’ leader sequence (Δ1-95)
FIG. 1. Identification of a conserved sequence upstream of *T. brucei* tRNAs. The coding and upstream sequences of the tRNAs from *T. brucei* were collected from the website http://zoosun00.unifr.ch/Trypanos/MITBIO.html (30) and from our own work (15). The *S. cerevisiae* coding and upstream sequences of the tRNAs were collected from the *Saccharomyces* Genome Database. Human tRNA sequences were extracted from the UCSC Genome Bioinformatics Database using the human BLAST search for sequences with homology to tRNAs of *T. brucei*. The resulting sequences were analyzed with tRNAScan-SE to predict the presence of tRNA genes (17). The 14-nucleotide upstream sequences plus tRNA coding sequences through position 37 were aligned for *T. brucei*, *Homo sapiens*, and *S. cerevisiae* with Clustal X. The sequences were adjusted manually with MacClade. The conservation at each residue was determined with Jalview. Conserved residues are highlighted as follows: dark blue, >80%; medium blue, >60%; light blue, >40%. The consensus sequence upstream of *T. brucei* tRNAs, YGG(C/A)RRC, was determined with MacClade. The highly conserved A box for each alignment is boxed, with the consensus sequence below. Invariant residues are shown in bold. Other universally conserved residues are indicated below the alignments. The anticodons for all tRNAs are boxed.
and further deletion of an additional 26 nucleotides (Δ1-121) had little effect on the level of import in comparison to the full-length dicistronic tRNA substrate, with import levels of 9.6, 8.8, and 7.5%, respectively (Fig. 2B). However, removal of the entire 5′ leader sequence in Δ1-154, including the YGG(C/A)RRC motif, reduced import to 1.9%. These results suggest that import of tRNALeu into the mitochondrion of T. brucei in vitro is affected by the presence of an element within the 5′ leader sequence, possibly acting as a positive determinant for import.

Precursor tRNA^Leu^ is found in both the mitochondrion and cytosol. Although our in vitro studies suggest that the 5′ flanking sequence influences mitochondrial import and a previous study showed that a dicistronic tRNA^Ser^•tRNA^Leu^ substrate containing the 5′ leader sequence was imported into the mitochondrion of T. brucei, the presence of the entire 5′ leader sequence is necessary for import.

FIG. 2. RNA import into isolated mitochondria and detection of precursor tRNA^Leu^ within trypanosome mitochondria. (A) Schematic of the precursor dicistronic tRNA substrate containing the 14-nucleotide 5′ flanking sequence, tRNA^Ser^, the 59-nucleotide intergenic region, tRNA^Leu^, and CCA, with the lengths of each 5′ deletion substrate shown beneath. The 5′ YGG(C/A)RRC motifs are shown upstream of both tRNA^Ser^ and tRNA^Leu^ (black boxes). All substrates were in vitro transcribed with T7 RNA polymerase in the presence of [α-32P]UTP for uniform labeling. (B) Mitochondria were incubated with 5 × 10^4 cpm of labeled substrate under optimized import conditions. The RNAs were collected by precipitation and treated with 30 μg of proteinase K per ml, which represents binding (lanes B). In the remaining reactions, proteinase K digestion was preceded by the addition of 10 U of micrococcal nuclease, followed by lysis of the mitochondria and extraction of the RNAs, which represents import (lanes I). The controls for resistance to micrococcal nuclease were first treated with 2% CHAPS before proteinase K and micrococcal nuclease treatment (lanes C). The isolated RNAs were run on a 6% polyacrylamide–8 M urea gel. The binding, import, and CHAPS lanes were quantitated with a phosphorimager. The percent import was determined as the counts per minute for the import lanes divided by the total counts per minute for import and binding. (C) A 27-nucleotide synthesized RNA oligonucleotide (black box) was ligated to mitochondrial and cytosolic RNA from pSer-Leu^+^ transfectants (see Fig. 3). The RNA was reverse transcribed with oligonucleotides 1 and 2. The resulting cDNAs were amplified with RNA oligonucleotide, complementary to the ligated RNA, and oligonucleotides 1, 2, and 3, separately. The PCR products were ligated into the TA vector and sequenced with T7 primer. The 5′ ends of mature tRNA^Leu^ from both mitochondria and cytosol were mapped, as indicated by the arrowhead. The 5′ ends of precursor tRNA^Leu^ from both mitochondria and cytosol were mapped and extended 14 nucleotides upstream of the mature 5′ ends.
LeBlanc et al. (15). These results indicate that tRNA\textsubscript{Leu} is synthesized as a precursor that can escape nuclear processing and be imported into the mitochondrion of \textit{T. brucei}. The unexpectedly high ratio of precursor to mature tRNA\textsubscript{Leu} in mitochondrial and cytosolic fractions is likely to be a consequence of preferential ligation of the RNA oligonucleotide to precursor tRNAs during 5\textsuperscript{\textprime}\text/H11032 RACE.

An in vivo system for analysis of tRNA localization. The presence of precursor tRNA\textsubscript{Leu} in mitochondrial and cytosolic fractions is likely to be a consequence of preferential ligation of the RNA oligonucleotide to precursor tRNAs during 5\textsuperscript{\textprime} RACE.

A poison primer extension assay was used to evaluate the abundance and localization of both endogenous tRNA\textsubscript{Leu} and tagged tRNA\textsubscript{Leu}\textsuperscript{\textasterisk} in mitochondrial (M) and cytosolic (C) RNA fractions with oligonucleotide LE4. Primer extensions were performed on 1 \textmu g of either mitochondrial or cytosolic RNA. This assay produces a 25-nucleotide product for endogenous tRNA\textsubscript{Leu}, present in both wild-type and pSer-Leu\textsuperscript{\textasterisk} transfectant control cells, and produces a 28-nucleotide product for the tagged tRNA\textsubscript{Leu}\textsuperscript{\textasterisk} in the pSer-Leu\textsuperscript{\textasterisk} transfected cells. (D) Deletion analysis. pSer-Leu\textsuperscript{\textasterisk}, tagged tRNA\textsubscript{Leu} with wild-type flanking sequences; pLeu\textsuperscript{\textasterisk}(\textsuperscript{+}), tagged tRNA\textsubscript{Leu} with upstream sequences, lacking both YGG(C/A)RRC motifs. (E) Poison primer extensions on 1 \textmu g of a mitochondrial (M) or cytosolic (C) RNA fraction in transfecants pSer-Leu\textsuperscript{\textasterisk}, pLeu\textsuperscript{\textasterisk}(\textsuperscript{+}), and pLeu\textsuperscript{\textasterisk}(\textsuperscript{-}) detect endogenous tRNA\textsubscript{Leu}, with a 25-nucleotide extension product, and tagged tRNA\textsubscript{Leu}\textsuperscript{\textasterisk}, with a 28-nucleotide extension product. The extension products were quantitated with a phosphorimager.
and cellular distribution. The purity of mitochondrial and cytosolic fractions was established by a poison primer extension assay to detect abundant markers for mitochondria and cytosol, 9S mitochondrial tRNA (9S tRNA) and spliced leader cytosolic RNA (Fig. 3B), respectively. Quantitation of these data indicated that contamination of mitochondrial and cytosolic RNAs was <3%. A poison primer extension assay detected the endogenous tRNALeu (25-nucleotide product) in both wild-type cells and a cell line transfected with pSer-Leu* (Fig. 3C). The tagged tRNA\textsuperscript{Leu*} (28-nucleotide product) was only detectable in the transfected cells (Fig. 3C).

Endogenous tRNA\textsuperscript{Leu} was localized in the mitochondrion and cytosol, with mitochondrial/cytosolic RNA ratios of 2.15 and 2.19 in wild-type and pSer-Leu* -transfected cells, respectively, showing that transfection and expression of the tagged tRNA\textsuperscript{Leu*} did not affect the cellular localization of endogenous tRNA\textsuperscript{Leu} (Table 1). The tagged tRNA\textsuperscript{Leu*} had a similar distribution between the mitochondrion and cytosol, with a mitochondrial/cytosolic RNA ratio of 1.70. Also, transfection and expression of the tagged tRNA\textsuperscript{Leu*} did not significantly alter the distribution of other endogenous tRNAs, since the mitochondrial/cytosolic RNA ratios of tRNA\textsuperscript{Met-e} were 3.58 and 3.33 in wild-type and pSer-Leu* -transfected cells, respectively (Table 1). Based on these results, it appears that cell fractionation allows for accurate determination of the localization of both tagged tRNAs and endogenous tRNAs within the cell.

\textbf{5’ flanking sequence influences abundance of tRNA\textsuperscript{Leu}.} The role of 5’ leader sequences of tRNA\textsuperscript{Leu} in mitochondrial localization was addressed by deletion of upstream sequences in vivo. We made a deletion construct from pSer-Leu* by deleting the upstream tRNA\textsuperscript{Ser} to the 5’ end of the mature tRNALeu. This deletion replaced the 5’ flanking sequence of tRNALeu with the 5’ flanking sequence from tRNA\textsuperscript{Ser} [Fig. 3D, pLeu*(+)]. Although the YGG(C/A)RRC motif (UGGCGGU) directly flanking tRNALeu was deleted, the same motif (UGGCGGU) flanks tRNACys; therefore, the motif was replaced upstream of tRNALeu. With pLeu*(+), the cellular localization and abundance of tRNALeu were unaffected (Fig. 3E), possibly due to retention of the sequence motif in the 5’ flanking sequence of tRNALeu. In contrast, removal of an additional 16 nucleotides, including the sequence motif, upstream of tRNALeu reduced the abundance of tagged tRNALeu by 96% in comparison to pSer-Leu* [Fig. 3E, pLeu*(−)]. Similar results were reported previously when 5’ flanking sequences were removed from tRNA\textsuperscript{Ser} and tRNALeu in T. brucei (11, 30). Unfortunately, the low abundance of tRNALeu observed with pLeu*(−) made it difficult to interpret the import results. However, these results suggest that the YGG(C/A)RRC motif within the 5’ leader sequence of tRNA\textsuperscript{Leu} is necessary for maintaining the cellular abundance of tRNALeu.

The YGG(C/A)RRC motif is involved in mitochondrial localization in vivo. The role of the YGG(C/A)RRC motif in the 5’ leader sequence of tRNA\textsuperscript{Leu} was further analyzed by mutations within the natural genomic context of the tRNA\textsuperscript{Ser}, tRNA\textsuperscript{Leu} gene cluster (Fig. 4A). Due to the severe effects on abundance that were observed when large deletions were made upstream of tRNALeu, we decided to make more conservative changes to the 5’ flanking sequence of tRNA\textsuperscript{Leu*}. Sequence replacement mutations were made in the YGG(C/A)RRC motif upstream of tRNA\textsuperscript{Leu*} (pSer-Leu* 1) and upstream of tRNA\textsuperscript{Ser} (pSer-Leu* 2). We mutated GGCGG to UCGCU, replacing the highly conserved dinucleotide GG. Mutation of the motif upstream of tRNA\textsuperscript{Leu*} decreased the mitochondrial localization of tRNA\textsuperscript{Leu} by 77% (Fig. 4B and E, pSer-Leu* 1). This mutation also resulted in a 73% reduction in the abundance of tRNA\textsuperscript{Leu*} within the cell (Fig. 4B, pSer-Leu* 1). Mutation of the motif upstream of tRNA\textsuperscript{Ser} in pSer-Leu* 2 did not negatively affect the localization of tRNA\textsuperscript{Leu} to the mitochondrion (Fig. 4B and E). Mutation of both motifs in the same construct gave the same result as that observed for pSer-Leu* 1 (data not shown). Again, the purity of mitochondrial and cytosolic fractions was established by detection of mitochondrial 9S tRNA and cytosolic spliced leader RNA for the cell lines transfected with pSer-Leu*, pSer-Leu* 1, and pSer-Leu* 2 (Fig. 4F). Quantitation of these data indicated that contamination of mitochondrial and cytosolic RNAs was <3%. These results show that the YGG(C/A)RRC motif in the 5’ flanking sequence directly upstream of tRNA\textsuperscript{Leu} is critical for maintaining normal cellular abundance and mitochondrial localization of tRNA\textsuperscript{Leu}.

Since the consensus YGG(C/A)RRC motif is not well conserved upstream of the cytosolically localized tRNA\textsuperscript{Met-i}, we replaced tRNA\textsuperscript{Leu*} in pSer-Leu* with tRNA\textsuperscript{Met-i}. Although the YGG(C/A)RRC motif within the 5’ leader of tRNA\textsuperscript{Leu*} showed evidence of mitochondrial localization by mutation with pSer-Leu* 1, addition of this sequence motif upstream of tRNA\textsuperscript{Leu*} did not result in mitochondrial localization of tRNA\textsuperscript{Met-i} (Fig. 4B and E; data not shown). This suggests that multiple signals may be required for efficient mitochondrial import.

\textbf{Mutation of the YGG(C/A)RRC motif affects the presence and localization of precursor tRNA\textsuperscript{Leu*}.} We performed 5’ RACE on mitochondrial and cytosolic fractions from pSer-Leu* and pSer-Leu* 1 transfectants, as shown in Fig. 2C, in order to determine whether accurate 5’ processing occurred for the tagged and mutated-tagged tRNA\textsuperscript{Leu*}, respectively. Interestingly, the localization of precursors for both the tagged tRNA\textsuperscript{Leu*} and endogenous tRNA\textsuperscript{Leu} was altered in the pSer-Leu* 1 transfectants. In pSer-Leu* transfectants, 40 clones were analyzed from the mitochondrial fraction that contained tRNA\textsuperscript{Leu}; 14 of these clones had tagged tRNA\textsuperscript{Leu*}, of which 7 had the 14-nucleotide 5’ leader sequence. The other seven had accurately processed, mature 5’ ends (Fig. 2C). In contrast, 5’ RACE of RNA from the mitochondrial fraction of pSer-Leu* 1 transfectants revealed a single endogenous precursor tRNALeu and no tagged precursor tRNALeu in a total of 27 tRNALeu clones.
FIG. 4. Mutation of the YGG(C/A)RRC motif in the 5’ flanking sequence of tRNA\textsuperscript{Leu*} influences localization of tRNA\textsuperscript{Leu*} and other mitochondrionally localized tRNAs. (A) Black boxes in pSer-Leu\textsuperscript{*} represent the sequence element GGC-GG, which is 4 nucleotides upstream of tRNA\textsuperscript{Leu*} and directly abuts tRNA\textsuperscript{Ser}. Open boxes in pSer-Leu\textsuperscript{*} 1 and pSer-Leu\textsuperscript{*} 2 denote mutations of the GGC-GG sequence element to UGC-GU. (B) Primer extension analysis of pSer-Leu\textsuperscript{*}, pSer-Leu\textsuperscript{*} 1, and pSer-Leu\textsuperscript{*} 2 was performed with oligonucleotide LE4 on 1 \( \mu \)g of mitochondrial (M) or cytosolic (C) RNA. The endogenous tRNA\textsuperscript{Leu} is detected by a 25-nucleotide extension product, and the tagged tRNA\textsuperscript{Leu*} is detected by a 28-nucleotide product. (C) Localization of endogenous tRNA\textsuperscript{Met-e} was determined by primer extension analysis with oligonucleotide Met e in pSer-Leu\textsuperscript{*}, pSer-Leu\textsuperscript{*} 1, and pSer-Leu\textsuperscript{*} 2. (D) Primer extension analysis with oligonucleotide Met i shows the localization of tRNA\textsuperscript{Met-i} in pSer-Leu\textsuperscript{*}, pSer-Leu\textsuperscript{*} 1, and pSer-Leu\textsuperscript{*} 2. (E) The ratio of mitochondrial/cytosolic RNA for tRNA\textsuperscript{Leu*}, endogenous tRNA\textsuperscript{Leu}, and tRNA\textsuperscript{Met-e} was analyzed for each transfec tant. This ratio was set to an import level of 1 for tRNAs from pSer-Leu\textsuperscript{*}, and the ratios for tRNAs from pSer-Leu\textsuperscript{*} 1 and pSer-Leu\textsuperscript{*} 2 were compared to that from pSer-Leu\textsuperscript{*} in graphical form (\( n = 6 \) for tRNA\textsuperscript{Leu*} and tRNA\textsuperscript{Leu}, \( n = 4 \) for tRNA\textsuperscript{Met-e}). (F) A poison primer extension assay was performed on 1 \( \mu \)g of a mitochondrial (M) or cytosolic (C) RNA fraction from the transfec tant lines pSer-Leu\textsuperscript{*}, pSer-Leu\textsuperscript{*} 1, and pSer-Leu\textsuperscript{*} 2. Oligonucleotide 9Sb produces a 22-nucleotide extension product, predominantly in the mitochondrial fraction. Oligonucleotide SL2 also produces a 26-nucleotide extension product, but it is primarily in the cytosol. Mitochondrial or cytosolic fractions with >3% cross-contamination were not used for subsequent experiments. SL RNA, spliced leader RNA.
Analysis of the cytosolic fractions also yielded unusual results for the tagged tRNA\textsuperscript{Leu}\textsuperscript{*} in pSer-Leu\textsuperscript{1} transfectants. As stated previously, 38 clones resulting from the cytosolic fraction of pSer-Leu\textsuperscript{1} transfectants contained tRNA\textsuperscript{Leu}\textsuperscript{*}, of the 8 clones that had the 14-nucleotide 5\textprime{} leader, 3 of the precursors were tagged tRNA\textsuperscript{Leu}\textsuperscript{*:}. Similarly, 24 clones resulting from the cytosolic fraction of pSer-Leu\textsuperscript{*} 1 transfectants contained tRNA\textsuperscript{Leu}\textsuperscript{*}, of which 12 were mature tRNA\textsuperscript{Leu} and 12 were precursor tRNA\textsuperscript{Leu} with a 14-nucleotide 5\textprime{} leader sequence. None of the 12 precursor tRNA\textsuperscript{Leu} was tagged, but there were two mature tagged tRNA\textsuperscript{Leu} clones. Unlike the mitochondrial and cytosolic fractions from pSer-Leu\textsuperscript{*} transfectants, there was no tagged precursor tRNA\textsuperscript{Leu}\textsuperscript{*} in either fraction from the pSer-Leu\textsuperscript{*} 1 transfectants. The lack of precursor tagged tRNA\textsuperscript{Leu}\textsuperscript{*} in pSer-Leu\textsuperscript{1} 1 transfectants is a sharp contrast to the results obtained with pSer-Leu\textsuperscript{*} transfectants. The reduced level of mitochondrial localization of tRNA\textsuperscript{Leu}\textsuperscript{*} and the corresponding lack of precursor tRNA\textsuperscript{Leu}\textsuperscript{*} within the pSer-Leu\textsuperscript{*} 1 transfectants implicate the 5\textprime{} leader sequence as an important component of mitochondrial localization (Fig. 4B and C).

Mutation in the YGG(C/A)RRC motif has global effects on cellular tRNA localization. The data thus far indicate the involvement of cis-acting sequences in the cellular localization of tRNA\textsuperscript{Leu}\textsuperscript{*}. Furthermore, closer examination of the data in Fig. 4 also reveals an effect on endogenous tRNA\textsuperscript{Leu} localization when the YGG(C/A)RRC motif is mutated (Fig. 4B and E, pSer-Leu\textsuperscript{*} 1). The ratio of endogenous mitochondrial/cytosolic tRNA\textsuperscript{Leu} was reduced by 41%, indicating that the distribution of tRNA\textsuperscript{Leu} between the mitochondrion and cytosol was altered. The reduction in tRNA\textsuperscript{Leu} localization to the mitochondrion is the same effect that was seen on the import level of transfected tRNA\textsuperscript{Leu}\textsuperscript{*} (Fig. 4B and E, pSer-Leu\textsuperscript{*} 1) and was dependent on mutation of the YGG(C/A)RRC motif upstream of tRNA\textsuperscript{Leu}\textsuperscript{*}. Mutation of the YGG(C/A)RRC motif upstream of tRNA\textsuperscript{Ser} did not have an effect on the localization of endogenous tRNA\textsuperscript{Leu} (Fig. 4B and E, pSer-Leu\textsuperscript{*} 2).

The effect of mutations to the YGG(C/A)RRC motif on endogenous tRNA\textsuperscript{Leu} led us to investigate whether other endogenous tRNAs are affected similarly. Poison primer extension assays on endogenous tRNA\textsuperscript{Met}c, a tRNA shared between both the cytosol and the mitochondrion, revealed that similar effects on mitochondrial localization occur for this endogenous tRNA as well. Mutation of the YGG(C/A)RRC sequence upstream of tRNA\textsuperscript{Leu}\textsuperscript{*} reduced the level of mitochondrial import for tRNA\textsuperscript{Met}c by 46% (Fig. 4C and E, pSer-Leu\textsuperscript{*} 1). Mutation of the YGG(C/A)RRC sequence upstream of tRNA\textsuperscript{Ser} did not result in a loss of import of tRNA\textsuperscript{Leu}\textsuperscript{*} or other endogenous tRNAs (Fig. 4B, to E, pSer-Leu\textsuperscript{*} 2). Other endogenous tRNAs that are shared between the mitochondrion and cytosol, including tRNA\textsuperscript{Tyr} (GUA) and tRNA\textsuperscript{Arg} (CCU), were also tested and gave similar results to those shown for the endogenous tRNA\textsuperscript{Leu} and tRNA\textsuperscript{Met}c (data not shown). Finally, no effect was seen on the cytosolic localization or abundance of the almost exclusively cytosolically localized tRNA\textsuperscript{Met}i with pSer-Leu\textsuperscript{*} 1 (Fig. 4D), suggesting that the effects caused by the YGG(C/A)RRC mutations are specific for localization of tRNAs to the mitochondrion.

DISCUSSION

We have identified a unique sequence motif, YGG(C/A)RRC, within the 5\textprime{} leader sequences of tRNAs in T. brucei. In this paper, we present evidence that the YGG(C/A)RRC sequence, immediately 5\textprime{} of the mature tRNA\textsuperscript{Leu} gene in T. brucei, influences both the abundance and localization of the resulting tRNA transcript, which contains this motif within its 14-nucleotide 5\textprime{} leader. Not only does mutating the 5\textprime{} flanking sequence of the tRNA\textsuperscript{Leu}\textsuperscript{*} transgene alter the localization of tRNA\textsuperscript{Leu}\textsuperscript{*} in the cell, but it also changes the localization of the endogenous tRNA\textsuperscript{Leu} and other mitochondrially localized tRNAs. Based on these results, and those of others, we conclude that tRNA import into trypanosome mitochondria is influenced by distinct sequence motifs present within precursor and mature tRNA sequences.

Sequences within a precursor mitochondrial tRNA influence tRNA abundance and cellular localization. Little is known about RNA polymerase III transcription in trypanosomes; however, the transcriptional start site of tRNA\textsuperscript{Ser} has been mapped by 5\textprime{} RACE to 14 nucleotides upstream of the mature 5\textprime{} end (15). We present evidence here from a 5\textprime{} RACE experiment that maps the transcriptional start site for tRNA\textsuperscript{Leu} to 14 nucleotides upstream of the mature 5\textprime{} end (Fig. 2C). Generally, transcription of a tRNA gene initiates at a purine within 10 nucleotides upstream of the mature tRNA coding sequence and continues until the polymerase reaches a cluster of thymidine residues, resulting in a precursor tRNA with short 5\textprime{} and 3\textprime{} flanking sequences (26, 27). Based on 5\textprime{} RACE studies, both tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Leu} from trypanosomes have extended 5\textprime{} leader sequences in comparison. Additionally, our 5\textprime{} RACE experiments have identified both precursor tRNA\textsuperscript{Leu} and tRNA\textsuperscript{Ser} with 14-nucleotide 5\textprime{} leaders within trypanosome mitochondria, suggesting that at least some tRNAs are imported into the mitochondrion as precursors (Fig. 2C) (15). These data appear to be contradictory to previously reported findings (1, 13). In those studies, it was shown that end processing precedes localization of tRNAs to the mitochondria of Leishmania tarentolae and that what appeared to be large precursor tRNAs in T. brucei and L. tarentolae mitochondria were artifacts generated by circularization of mature tRNAs (1, 13). Kapushoc et al. used an RT-PCR assay to show that precursor tRNAs with 29- to 34-nucleotide 5\textprime{} and/or 3\textprime{} extensions are only localized in the nucleus, but they did not look specifically for precursor tRNAs with a 5\textprime{} leader sequence (13). While we have reported the presence of a dicistronic tRNA\textsuperscript{Ser}-tRNA\textsuperscript{Leu} in the mitochondrion, our 5\textprime{} RACE data with tRNA\textsuperscript{Leu} show that precursor tRNA\textsuperscript{Leu} with a 14-nucleotide 5\textprime{} leader sequence is much more abundant in the cell. Furthermore, the genomic organization of the trypanosome genome, with singular tRNAs, head-to-head tRNAs, tail-to-tail tRNAs, and head-to-tail tRNAs, would not allow for all tRNAs to be transcribed as multicistronic transcripts (15, 30). We agree that the large tRNA molecules found in mitochondrial fractions are mostly due to ligation of mature tRNAs during our mitochondrial isolation procedure (15).

Based on the mapping of the transcription initiation site to 14 nucleotides upstream of the mature tRNA\textsuperscript{Leu}, we performed detailed sequence alignments from –14 nucleotides upstream of the mature tRNAs to position 37 within the cod-
ing sequence of 23 T. brucei tRNA genes (Fig. 1). These alignments revealed a conserved sequence motif upstream of the tRNAs with a consensus of YGG(C/A)RRC. We compared the T. brucei upstream sequences to those from human and S. cerevisiae tRNAs. While S. cerevisiae imports a single tRNA, the mechanism is different from that in kinetoplastids (24, 31). All of the human mitochondrial tRNAs are encoded by the mitochondrial genome, although human mitochondria are able to import tRNA from S. cerevisiae in the presence of yeast cytosolic factors (14). The alignment of upstream regions of human or S. cerevisiae tRNAs failed to reveal a conserved sequence element, although the regions upstream of S. cerevisiae tRNAs are very A rich. The identification of a conserved motif upstream of T. brucei tRNA genes supported the possibility that sequences within the 5′ leader of precursor tRNAs in T. brucei might be important for tRNA localization.

Initial evidence for the functional significance of the 5′ leader sequence in mitochondrial import came from in vitro import studies. We found that deletion of sequences containing the YGG(C/A)RRC motif, directly upstream of tRNA, reduced the efficiency of tRNA import (Fig. 2B). In order to evaluate the role of this sequence motif in vivo, we developed a transfection assay for tRNA import and abundance in T. brucei (Fig. 3). A single nucleotide change in the variable arm of tRNA allowed us to distinguish the transfected tRNA from endogenous tRNA and to determine the steady-state levels of tRNA in the cytosol and mitochondrion. Using this assay, we showed that a deletion of 156 nucleotides of the tRNA 5′ flanking sequence in pSer-Leu+ [pLeu*(-)] included the upstream tRNA and both YGG(C/A)RRC motifs, resulted in a severe decrease in the abundance of tRNA (Fig. 3E). To determine whether the decrease in tRNA abundance was due to deletion of the YGG(C/A)RRC motif, we replaced the motif immediately upstream of tRNA in construct pLeu+ (Fig. 3D). This restored the abundance and mitochondrial localization of tRNA to the same level as that seen for pSer-Leu+ (Fig. 3E), presumably because the motif located directly 5′ of tRNA was juxtaposed with the tRNA gene [Fig. 3D and E, pLeu+(+)]. Unfortunately, the low abundance of tRNA observed with pLeu*(-) made it difficult to interpret the import results. The observed decrease in abundance could result from a number of situations, including transcript stability, nuclear export, or transcription. Taken together, these in vitro and in vivo results support that the YGG(C/A)RRC motif within the 5′ leader sequence of tRNA influences the abundance and localization of tRNA in the mitochondrion.

Since large deletions of the 5′ flanking sequence had a severe effect on tRNA abundance, we decided to prepare more limited mutations by scrambling the sequence of each YGG(C/A)RRC motif of tRNA and tRNA independently (Fig. 4A). We mutated GCCGG in the motif to UCGCU, changing the highly conserved dinucleotide GG to pyrimidines (UC), the central C residue to a purine (G), and the conserved RR residues to pyrimidines (GG to CU). Although mutating the YGG(C/A)RRC motif upstream of tRNA did not affect the localization of tRNA (pSer-Leu 2), mutation of the YGG(C/A)RRC motif upstream of tRNA (pSer-Leu 1) decreased its localization to the mitochondrion significantly (Fig. 4B and E). A striking consequence of this mutation was discovered in 5′ RACE experiments on both pSer-Leu and pSer-Leu transfectants. In trying to determine whether the mutation caused an alteration in accurate 5′ processing of tRNA, which it did not, we found a severe reduction in the amount of precursor tRNA in the mitochondria of pSer-Leu transfectants and no precursor tRNA in these cells. While tRNA was accurately 5′ processed, the lack of precursor tRNA with a 5′ leader suggests two possibilities: the mutation may lead to more efficient 5′ processing so that we are unable to detect these precursors in the cell or transcription may initiate at the site of the mature 5′ end of tRNA. If the second possibility occurs, trypanosomes may have the ability to import both mature and precursor tRNAs into the mitochondrion.

At first glance, these data appear to contradict published studies on mitochondrial tRNA import in kinetoplastids. Others have reported that the genomic context of tRNA genes has no influence on their localization to the mitochondrion (16, 30). However, upon reevaluation of published data, it appears that the 5′ flanking sequence influences tRNA localization in experiments with Leishmania. The 5′ flanking sequence of tRNA, a tRNA localized in the cytosol and mitochondrion, was interchanged with the 5′ flanking sequence from a cytosolically localized tRNA or plasmid sequence. This change decreased the efficiency of localization of tRNA to the mitochondrion (16). This is consistent with the results we present here. Studies in T. brucei using tRNA showed progressive deletions of the 5′ flanking sequence, with no effect on import (30). Yet, in the experiment in which the YGG(C/A)RRC motif was removed, the abundance of tRNA was reduced such that localization of the tRNA into the mitochondrion was difficult to interpret. Our interpretation of the results of Lima and Simpson supports our conclusion that sequences within the 5′ leader sequence of precursor tRNAs influence the efficiency of import, and the results of Tan et al. do not provide sufficient evidence for the conclusion that the 5′ flanking sequence does not influence mitochondrial localization (16, 30).

**Multiple sequence motifs influence tRNA import into kinetoplast mitochondria.** Mutation of the YGG(C/A)RRC sequence within the 5′ leader of tRNA lowers the efficiency of import but does not abolish import of tRNA into T. brucei mitochondria (Fig. 4B). Therefore, other elements within the mature tRNA sequence must influence localization. In Leishmania, exchanging the D loop and stem of cytosolic tRNA with those of mitochondrially imported tRNA changes the localization of the hybrid tRNA so that it imports into mitochondria (16). Yet, exchanging the D loop and stem of the shared tRNA with those of cytosolically localized tRNA did not exclude the hybrid tRNA from mitochondria (16), suggesting the presence of multiple signals within either the mature tRNA sequence or the 5′ leader sequences.

The diversity of sequence and structural motifs that may be involved in tRNA trafficking in trypanosomes was demonstrated by SELEX experiments in which oligoribonucleotide aptamers were selected based on their efficiency of import into Leishmania mitochondria (4). Interestingly, these studies identified the sequence YGGYAGAGY as a positive import signal, and candidate sequences that fit this consensus were identified in the D arm, Tu arm, or variable arm of mitochondrially imported Leishmania tRNAs (4). This sequence is highly ho-
mologous to the YGG(C/A)RRC motif that we have identified upstream of tRNA genes for *T. brucei*.

We replaced the 5′ leader sequence of the cytosolically localized tRNA^Met^ with that of tRNA^Aeu^, which has a highly conserved YGG(C/A)RRC motif, in order to determine whether this motif is sufficient to direct mitochondrial localization of tRNA^Met^. The motif did not direct mitochondrial localization of tRNA^Met_, possibly because this tRNA lacks the homologous to the YGG(C/A)RRC motif that we have identified upstream of tRNAs to mitochondria. Adding a further localization of tRNAMet-i, possibly because this tRNA lacks the redundant signals for mitochondrial localization since they are necessary for mitochondrial protein translation.

**Mutation of the YGG(C/A)RRC motif has a global effect on tRNA import.** A surprising result from our studies is that not only does mutation of the upstream YGG(C/A)RRC motif alter the localization of tRNA^Aeu_, but it also alters the mitochondrial abundance of other endogenous tRNAs (Fig. 4, pSer-Leu^+1 transfectants). This brings up the question of where mitochondrial and cytosolic localization diverge during tRNA biogenesis. Does sorting take place in the nucleus, in the cytoplasm, or at the mitochondrial membranes? Alternatively, do the multiple signals contained within the precursor tRNAs influence localization at separate points in the pathway? While our studies do not directly address the mechanism of import, the global effect we observed on tRNA localization when the YGG(C/A)RRC motif of tRNA^Leu^ was mutated suggests that this sequence may be involved at a later step in the trafficking of tRNAs to mitochondria.

While previous studies showed the presence of a dicistronic tRNA precursor in the mitochondrion of *T. brucei* (15), our data with the monocistronic pLeu^*+1 cell line conclusively show that this is not the only substrate for tRNA localization to the mitochondrion. Also, the genomic organization of the clustered tRNA genes in *T. brucei* as head-to-head or tail-to-tail, as well as the presence of nonclustered tRNAs, would not allow for all tRNAs to be transcribed as dicistronic or multicistronic transcripts (20, 30). Although many questions remain concerning the mechanism of tRNA localization to the mitochondrion in *T. brucei*, we have presented evidence here of a unique sequence motif found within the 5′ leader sequence of a precursor tRNA that influences both the cellular localization and abundance of tRNA^Leu_, suggesting a role for precursor tRNAs in the tRNA localization pathway to the mitochondrion. Furthermore, the motif we have identified upstream of trypanosome tRNAs is unique as an extragenic element that modulates both abundance and localization of tRNAs. In addition, we have shown that mutation of the YGG(C/A)RRC motif, upstream of tRNA^Aeu_, causes a global change of tRNA localization to the trypanosome mitochondrion, adding a further level of interest to this unique sequence motif.

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