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Are tRNAs imported into the mitochondria of kinetoplastid protozoa as 5′-extended precursors?

Ruslan Aphasizhev [a,*], Uma Karmarkar [a], Larry Simpson [a,b]

[a] *Howard Hughes Medical Institute, UCLA, 6780 MacDonald Building, Los Angeles, CA 90095-1662, USA*
[b] *Departments of Molecular, Cell and Developmental Biology and Medical Microbiology and Immunology, UCLA, 6780 MacDonald Building, Los Angeles, CA 90095-1662, USA*

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Abstract

All mitochondrial tRNAs in kinetoplastid protozoa are encoded in nuclear DNA and transported into the mitochondrion (Simpson et al., Nucl Acids Res 1989;17:5427–5445; Hancock and Hajduk, J Biol Chem 1990;265:19208–19215). It has been proposed that tRNAs in these cells are imported into the mitochondrion as 5′-extended precursors which are processed by a mitochondrial RNase P-like activity (Hancock et al., J Biol Chem 1992;267:23963–23971). We have examined this hypothesis by cloning and sequencing primer extension products of mitochondrial tRNAs from *Leishmania tarentolae* and *Trypanosoma brucei*, and have found that these are derived from circularized mature tRNA molecules. We suggest that these molecules are produced by the endogenous RNA ligase activity (Bakalara et al., J Biol Chem 1989;264:18679–18686) either in vivo or during mitochondrial isolation. We did not obtain any evidence for the existence of high molecular weight precursors of mitochondrial tRNAs. This negative result is consistent with previous in vivo transfection studies with both *L. tarentolae* (Lima and Simpson, RNA 1996;2:429–440) and *T. brucei* (Hauser and Schneider, EMBO J 1995;14:4212–4220; Schneider et al., Mol Cell Biol 1994;14:2317–2322), in which mitochondrial targeting of plasmid-expressed tRNAs was independent of the presence of 5′-flanking sequences. We conclude that the hypothesis for 5′-extended tRNA precursors in kinetoplastid mitochondrial importation remains to be verified.

Keywords: Mitochondrial tRNA; Kinetoplastid protozoa; *L. tarentolae*; *T. brucei*

1. Introduction

In animals, the tRNAs that function in mitochondrial translation are all encoded in the mitochondrial genome. However, in a variety of other organisms, ranging from higher plants to yeast and protozoa, a variable number of mitochondrial tRNAs are encoded in the nuclear genome and transported into the mitochondrion. A single nuclear-encoded $tRNA^{Lys}$(CUU) of unknown func-

* Corresponding author.

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tion in baker's yeast mitochondria [1], is transported into mitochondria in vivo and in vitro, and the importation is dependent on energy, an electrochemical membrane potential, several known inner and outer membrane receptor proteins, and cytosolic fractions which include the cytosolic aminoacyl tRNA synthetases [1–4]. All mitochondrial tRNAs in the kinetoplastid protozoa, *Leishmania tarentolae* and *Trypanosoma brucei*, are encoded in nuclear DNA and apparently imported [5,6]. In vitro importation of small synthetic RNAs and tRNAs into mitochondria from *Leishmania tropica* by a protein receptor-mediated pathway has been reported [7–9], but unlike the yeast importation system, no cytosolic factors were required.

In vivo targeting experiments in *L. tarentolae* showed that swapping the D-loop + stem of a mitochondrial-localized $tRNA^{Ile}$(UAU) conferred a partial mitochondrial localization on a normally solely cytoplasmic $tRNA^{Gln}$(CUG) [10], suggesting the presence of a mitochondrial importation signal within this region of the molecule. Short RNAs possessing a purine-rich sequence motif were reported to be good substrates for in vitro mitochondrial importation in *L. tropica* [8]. In *T. brucei*, several episomally-expressed tRNAs, even mutants incapable of aminoacylation and heterologous tRNAs from other species, were imported efficiently in vivo [11]. In *Tetrahymena*, the anticodon has been identified as a signal sequence for mitochondrial import of glutamine tRNA in vivo [12], but in *T. brucei*, an intron-containing tRNA with an altered anticodon loop structure was reported to be imported as efficiently as the native tRNA [13].

Hancock et al. [14] proposed that tRNAs in *T. brucei* are imported as precursor molecules with 5′ extensions ≈ 100 nucleotides in length, which was then cleaved off by an RNase P-type activity within the mitochondrion [14]. However, in vivo evidence in the *L. tarentolae* [10], *T. brucei* [11] and yeast [15] systems suggested that 5′ flanking genomic sequences do not play a role in mitochondrial targeting of nuclear DNA-encoded or plasmid-encoded tRNAs. In these systems, the tRNA structure itself contained necessary and sufficient information for mitochondrial import. In order to address this discrepancy, we attempted to clone and sequence mitochondrial-specific 5′-extended tRNA precursors.

2. Materials and methods

2.1. Metabolic labeling of purified mitochondria from L. tarentolae

Mitochondria (2.5 mg/ml) were incubated at in 50 mM HEPES (pH 7.4), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.2 M of sucrose and 0.5 mM ATP for 30 min at 27°C to deplete the endogenous pool of nucleotides. [α-^{32}P]NTP (100 μCi/ml) was added and incubation continued for 1 h. RNA was extracted and analyzed on a 10% acrylamide/7 M urea gel.

2.2. Northern blot analysis

After electrophoresis in acrylamide/urea gel, the *L. tarentolae* kinetoplast and total RNAs were electroblotted to a 0.2 μm Nytran Plus membrane (Schleicher & Schuell) and hybridized with the following probes: S1447: TGCTCCCGGCGGGGCTCGAACCCGCGACATCCGGTTCATAAGACCAGCGTCCGTGCCAACT AGA (64-mer, antisense of $tRNA^{Ile}$); S1687: CACTCCTACCTGGACTCGAACCAGGGTTATCGGAT TCAGAAGTCCGAGGTGAGTTCCGCTA CAC (63mer, antisense of $tRNA^{Gln}$). Hybridization was in 5 × SSPE, 5 × Denhardt's, 0.2 mg/ml denatured sonicated salmon sperm DNA, 0.5% SDS at 55°C, followed by washes in 0.2 × SSPE, 0.1% SDS at 55°C. The radioactive bands were visualized in a PhosphoImager (Molecular Dynamics).

2.3. Primer extension analysis and cloning of extension products

Mitochondrial RNA was isolated from a purified kinetoplast fraction as described previously [16]. The purified mitochondrial RNAs were routinely checked for cytoplasmic RNA contamination, which was $< 5\%$. Mitochondrial RNA (50 μg) was hybridized with 0.5 pmol of 5′-labeled

primer in 30 μl of 40 mM PIPES, pH 6.0, 0.4 M NaCl, 80% formamide at 40°C overnight and ethanol-precipitated. The pellet was dissolved in 20 μl of 1 × RT buffer (Promega) with 0.5 mM dNTP mixture, and incubated with 10 U of AMV reverse transcriptase (Promega) for 1 h at 45°C. RNA was digested with RNase A, and extension products were precipitated and analyzed on 10% sequencing gel. Bands of interest were eluted and subjected to 5′-RACE, using G-tailing with terminal deoxynucleotide transferase according to the manufacturer's protocol (Boehringer). Primers used were S2269: GTCCGAGGTGATAACCGCTACAC for $tRNA^{Gln}$ of *L. tarentolae*, S2253: CGTCTTAGACCACTCGACCATC for $tRNA^{Leu}$ of *T. brucei*, oligo$[C]_{15}$.

3. Results

3.1. Presence of slowly migrating mitochondrial tRNAs in acrylamide gels

It was shown previously that $tRNA^{Ile}$(UAU) in *L. tarentolae* is mainly localized in the mitochondrial fraction and $tRNA^{Gln}$(CUG) is mainly localized in the cytosolic fraction [10,17]. As shown in Fig. 1, two minor bands containing $tRNA^{Ile}$ sequences were detected by Northern blot analysis of *L. tarentolae* mitochondrial RNA but not of cytosolic RNA. These bands migrate slower than the major tRNA band in 12% acrylamide/7 M urea and have apparent sizes of ≈170 and 600 nucleotides. A $tRNA^{Gln}$-specific 170 nt band was also observed, but this was of a lower intensity. The 170 nt band was 0.2–0.8% of the intensity of the tRNA band, regardless of the amino acid specificity of the tRNA. It is known that the $tRNA^{Gln}$(UGG) isoacceptor has a partial mitochondrial localization [10] and the oligonucleotide probes used probably hybridize with all isoacceptors, thereby reflecting the distribution of the total $tRNA^{Gln}$ and $tRNA^{Ile}$ pool. It was not possible to detect any slowly migrating tRNA signals when a 2% agarose/formaldehyde gel was used for RNA separation (data not shown).

The apparent molecular weights of the tRNA-specific upper bands varied with the gel concentration. The Northern blots in Fig. 2a and b show that the tRNA-specific band which migrates with an apparent molecular weight of 170 nt in 12% acrylamide, migrates with an apparent molecular weight of 130–140 nt in 6% acrylamide and 200–220 nt in 16% acrylamide. A variation of apparent molecular weight with gel concentration is characteristic of circular [18] or highly structured molecules [19]. The circular nature of the 170 nt band was confirmed by subjecting the gel-isolated RNA to partial alkaline hydrolysis, which resulted in the release of full length linear tRNA with no degradation products between the 170 nt band and the tRNA band, as shown in Fig. 3c.

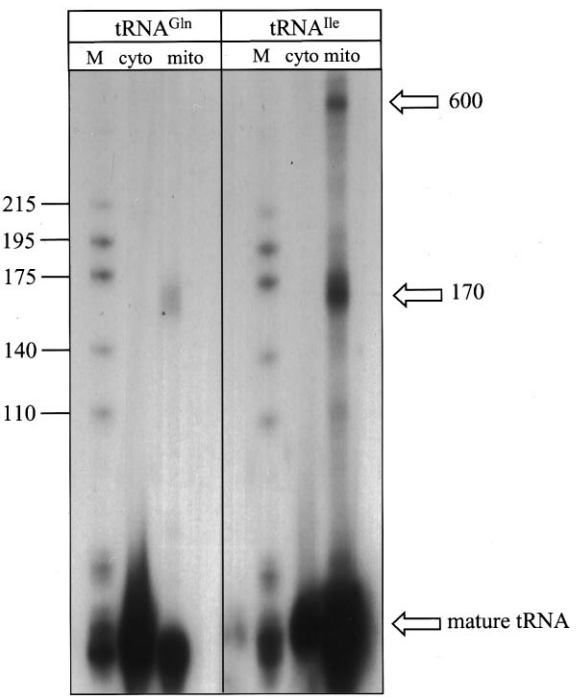

Fig. 1. Northern blot analysis of *L. tarentolae* RNA with tRNA-specific oligonucleotide probes. RNA was isolated from total cells (cyto) or purified mitochondria (mito) and electrophoresed in 12% acrylamide/7 M urea. The electroblots were hybridized with [^{32}P]-5′-labeled S1447 oligonucleotide (antisense to $tRNA^{Ile}$), and S1687 oligonucleotide (antisense to $tRNA^{Gln}$), respectively. The 170 and 600 nt tRNA-specific upper bands are indicated by arrows. The size standards (M) are small rRNAs from *L. tarentolae* 3′-labeled with [^{32}P]Cp and RNA ligase.

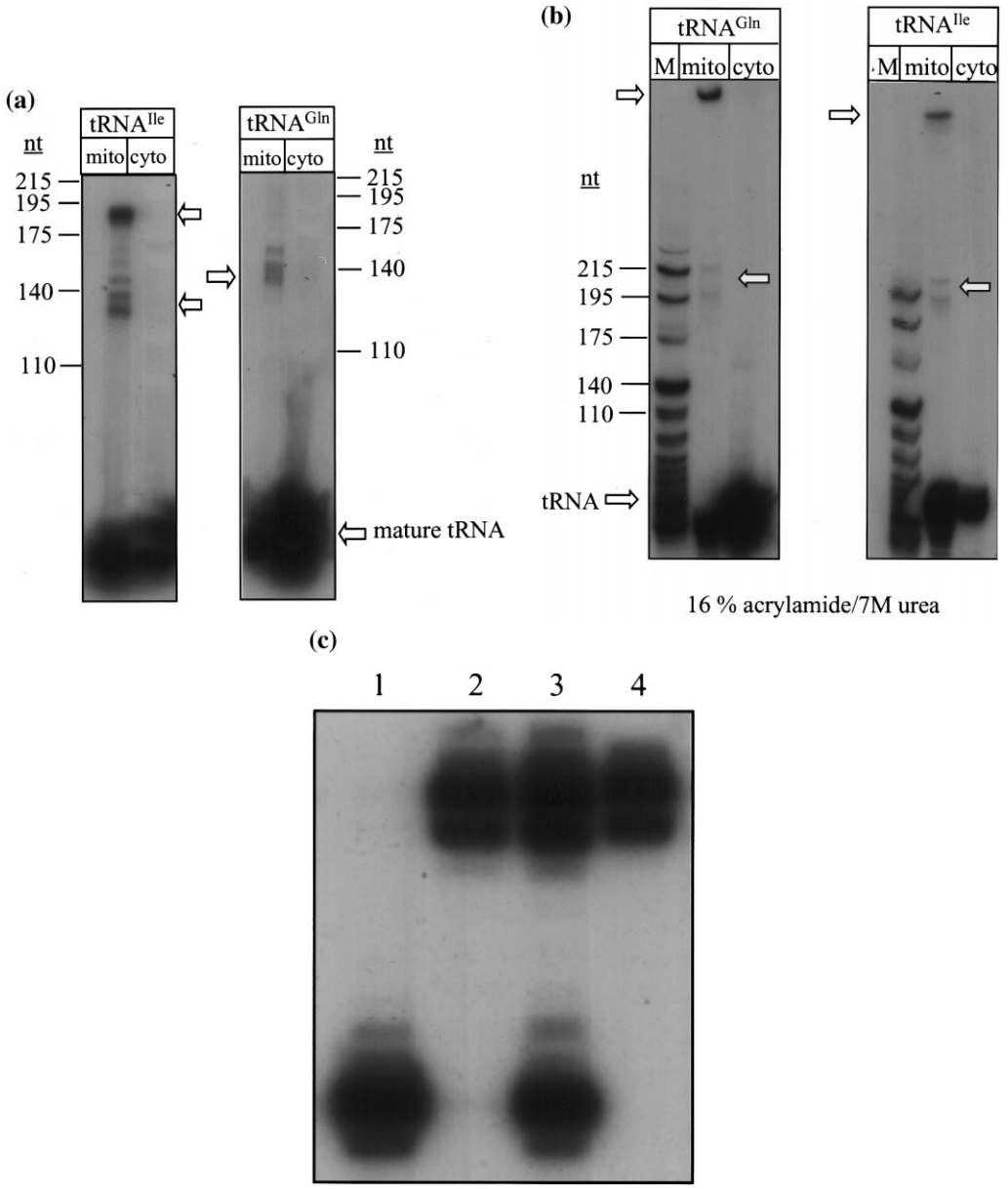

Fig. 2. Effect of gel concentration on apparent molecular weight of the tRNA-specific upper bands. Cytoplasmic (cyto) and mitochondrial (mito) RNAs were electrophoresed in (a) 6% and (b) 16% acrylamide/7 M urea, and the electroblots hybridized with the same probes as in Fig. 1. The tRNA-specific upper bands are indicated by arrows. (c) Partial alkaline treatment of gel-isolated upper bands. Lane 1, $tRNA^{Ile}$ (concentration adjusted to give a signal comparable with that of upper band. Lane 3, same as in lane 2, but incubated with 20 mM NaOH at 65°C for 5 min. Lane 4, same as in lane 3, but NaOH omitted.

3.2. Metabolic labeling of mitochondrial tRNAs with [α-^{32}P]CTP also identifies two minor slowly migrating tRNA fractions

An isolated mitochondrial fraction from *L. tarentolae* was incubated with [α-^{32}P]GTP and [α-^{32}P]CTP after pre-incubation for depletion of endogenous nucleotides. The absence of any labeled species after incubation with [α-^{32}P]GTP in the gel shown in Fig. 3 suggests a lack of tran-

scription in these nucleotide-depleted mitochondria. Incubation with [α-^{32}P]CTP yielded two minor high molecular weight labeled RNA bands of $\approx$160–180 and 500–600 nt, which apparently result from the 3′ terminal addition of CC(A) residues by a mitochondrial tRNA nucleotidyltransferase activity (Fig. 3). The heterogeneity of the bands probably mirrors the heterogeneity of the total mitochondrial tRNA population. These

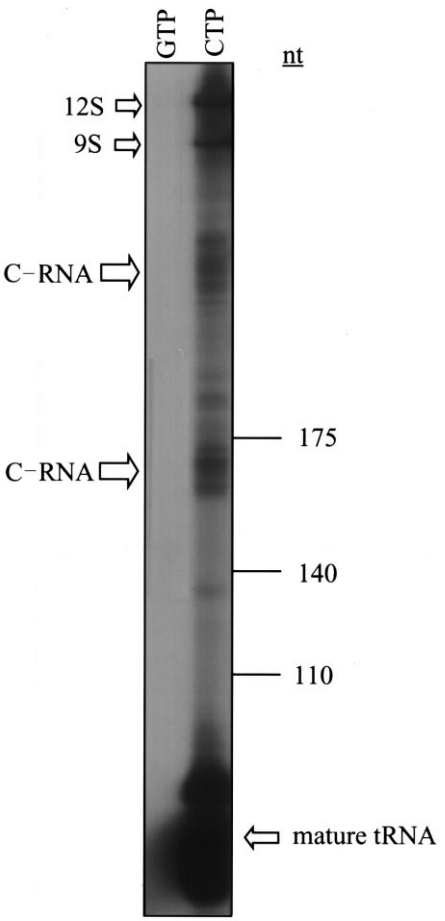

Fig. 3. Metabolic labeling of purified mitochondria from *L. tarentolae*. Mitochondria were pre-incubated to deplete the endogenous nucleotide pools as described in Section 2, and were then incubated with the radioactive nucleotides indicated for 60 min at 27°C. RNA was isolated and analyzed on a 10% acrylamide/7 M urea gel. The two slowly migrating CTP-labeled bands are indicated as 'C-RNA'. The positions of the 9S and 12S mitochondrial rRNAs are also indicated.

bands appear to be identical to the two slowly migrating circular tRNA-specific bands identified above by Northern hybridization. Our attempts to detect high molecular weight tRNA-containing molecules, either by 3′-end labeling with [^{32}P]pCp and T4 RNA ligase or 5′-end labeling with [γ-^{32}P] and T4 polynucleotide kinase were unsuccessful (data not shown). Labeling of ribosomal RNAs with a mitochondrial tRNA nucleotyltransferase activity may reflect either a somewhat degenerate specificity of this enzyme, or the presence of tRNA-like structures which mimic properties of the natural substrates [20].

3.3. Use of primer extension analysis to identify 5′-extended tRNA precursors

Primer extension analysis was performed using oligonucleotides complimentary to the anticodon or D-hairpin regions of tRNA^{Gln} and tRNA^{Ile} from *L. tarentolae*, and tRNA^{Leu}, tRNA^{Asn}, tRNA^{Lys} and tRNA^{Arg} from *T. brucei*. Multiple minor extension products of different sizes were obtained, as shown for the *L. tarentolae* tRNA^{Gln} and the *T. brucei* tRNA^{Leu} in Fig. 4. It should be noted that the extension products of +28, +53 and +224 (the mature 5′ end of tRNA is considered +0) obtained for the *T. brucei* tRNA^{Leu} differed from the approximately +125 product described previously by Hancock et al. [14]. The bands indicated by arrows in Fig. 4 were eluted from the gel, 3′-tailed with G residues, PCR amplified using an oligo[C] primer and the identical tRNA-specific primer used for the primer extension and the PCR products were cloned and sequenced. RT-PCR amplification of the extension product for *L. tarentolae* tRNA^{Ile} was unsuccessful.

Sequence analysis indicated that the +53 nt and +224 nt extension products for tRNA^{Leu} from *T. brucei* were actually fragments of the 12S mitochondrial rRNA (data not shown). The origin of these products appears to be due to mispriming during the reverse transcription step.

The +45 nt extension product for the *L. tarentolae* tRNA^{Gln} and the +28 nt extension product for the *T. brucei* tRNA^{Leu} proved to be derived from circularized tRNAs. As shown in Fig. 5, the

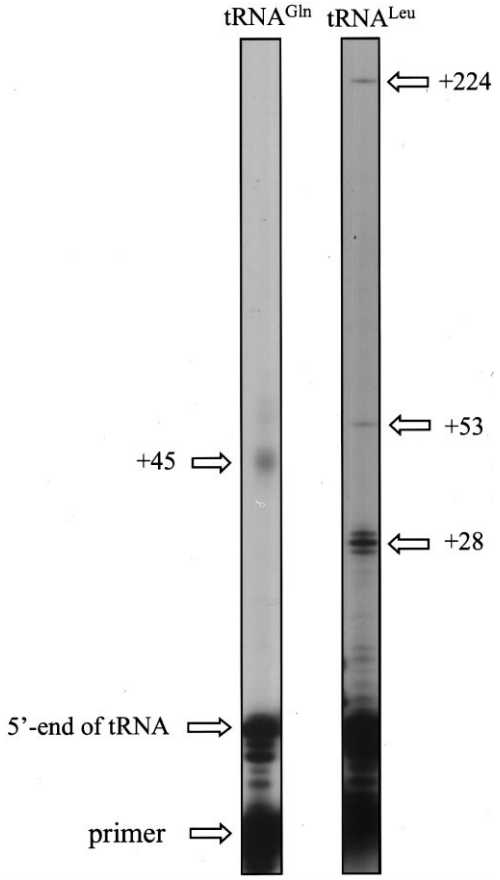

Fig. 4. Primer extension analysis of *L. tarentolae* $tRNA^{Gln}$ and *T. brucei* $tRNA^{Leu}$ using total mitochondrial RNA from each species. The sizes of specific high molecular weight extension products discussed in the text are indicated by arrows, as are the localizations of the 5′ ends of the tRNAs and the primers.

5′ tRNA sequence was joined to the 3′ sequence in each case, suggesting that these bands were derived from self ligation of these mitochondrial tRNAs, probably by the known mitochondrial RNA ligase [21,18]. The mature tRNA 5′ end was observed in all clones sequenced (12 clones for $tRNA^{Gln}$ and 17 clones for $tRNA^{Ile}$), while the 3′ end of the tRNA was heterogeneous for the last three (CCA) nucleotides.

4. Discussion

Hancock et al. [14] previously showed that a minor class of mitochondrial RNAs in *T. brucei*, which were metabolically labeled in isolated mitochondria by incubation with [α-^{32}P]CTP, migrated more slowly than mature tRNAs in acrylamide gels. A single heterogeneous so-called 'C-RNA' band with an apparent size of $\approx$120–180 nt was observed in 6% acrylamide, and in 12% acrylamide two bands migrating with apparent sizes of 165–190 and 500–735 nt were observed. Minor bands with the same mobility also appeared when total mitochondrial RNA was labeled with yeast nucleotidyltransferase and [α-^{32}P]CTP. The metabolically-labeled C-RNAs showed hybridization to cloned nuclear tRNA genes. These authors also showed that digestion of gel-isolated metabolically labeled C-RNAs with *E. coli* or yeast RNase P or with a *T. brucei* mitochondrial extract produced products that migrated at a position commensurate with the size of mature tRNA. Primer extension products migrating with an apparent size commensurate with 5′-extended tRNAs were also detected using oligonucleotides complementary to $tRNA^{Asn}$ and $tRNA^{Leu}$.

The metabolic labeling and the primer extension data were interpreted as evidence for the existence of 5′-extended tRNA precursors in total mitochondrial RNA. The authors [14] suggested that these precursors represented the substrate for tRNA importation, which was followed by cleavage of the 5′ extension by an RNase P-like activity. However, the facts that C-RNAs were not a substrate for [^{32}P]pCp 3′-end addition by T4 RNA ligase, nor for [γ-^{32}P]ATP 5′-end addition by T4 polynucleotide kinase, and the fact that the apparent size of the C-RNAs varied with the gel concentration are more consistent with these being circular molecules rather than 5′-extended linear tRNAs. In addition, Hancock and Hajduk [22] found that it was not possible to identify a 5′-end extended tRNA in *T. brucei* by hybridization with a probe complementary to the genomic sequence immediately upstream of the $tRNA^{Asn}$ (GUU) gene. We also could not detect 5′-extended $tRNA^{Ile}$ and $tRNA^{Gln}$ in *L. tarentolae* by hybridization with genomic probes (data not shown). Nor was the existence of any 5′-extended precursor tRNAs ever confirmed by cloning and sequencing in the original report [14]. Hauser and Schneider [11] also detected a mitochondrial-

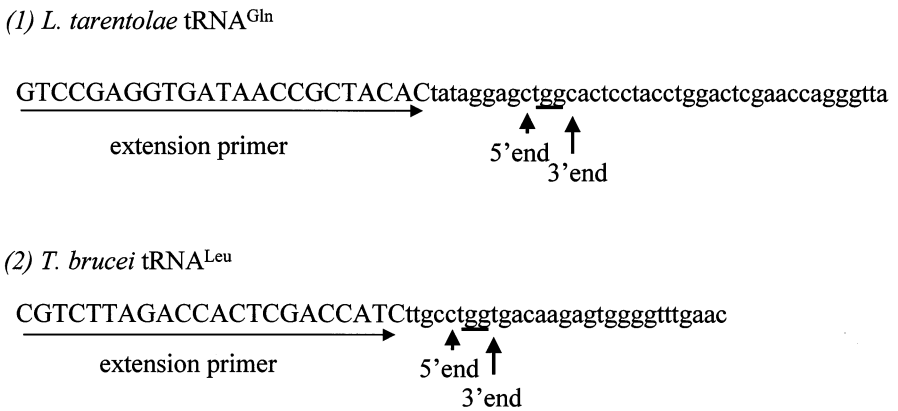

Fig. 5. Partial sequences of cloned PCR products derived from primer extensions of the *L. tarentolae* $tRNA^{Gln}$ and the *T. brucei* $tRNA^{Leu}$. The localizations of the primers used for both PCR and sequencing are indicated by underline arrows. The 5′ ends and the 3′ ends of the tRNA are shown by arrows. The observed linkage of the 5′ end with the 3′ end is indicative of a circularized tRNA molecule.

localized slowly migrating form of a plasmid-expressed $tRNA^{Tyr}$ in *T. brucei*, but showed that this occurred with or without the presence of a 5′-flanking sequence, and that a probe specific for the immediate 5′-flanking genomic region of this tRNA failed to hybridize with the slowly migrating band in Northern analysis.

We have partially reproduced the original observations of Hancock et al. [14] with *T. brucei* tRNA, with the additional step of cloning and sequencing the primer extension products. We have also searched for high molecular weight tRNA precursors in *L. tarentolae* mitochondrial fractions. We have not obtained evidence for 5′-extended tRNAs in either system. Two high molecular weight primer extension products obtained with *T. brucei* $tRNA^{Leu}$ represented mispriming on rRNA. Two other primer extension products obtained with *T. brucei* $tRNA^{Leu}$ and *L. tarentolae* $tRNA^{Gln}$ were shown by sequence analysis to be derived from circularized mature tRNAs. The abnormal gel mobilities of the slowly migrating tRNA-containing bands which we detected by Northern analysis are also consistent with these molecules being circular tRNAs. We speculate that the previously observed production of mature tRNA-sized molecules in *T. brucei* by digestion of the metabolically-labeled C-RNA with heterologous RNase P or with a *T. brucei* mitochondrial extract [14] could have been due to nuclease cleavage of circular molecules, which would release linear molecules with the size of a mature tRNA.

Our sequence analysis of cloned tRNA primer extension products also indicates that mitochondrial tRNAs have mature 5′ termini, In addition, since 3′ termini ending in either C, CC or CCA were observed in different clones, it follows that CCA addition occurs in vivo within the organelle, as was previously suggested from in organellar [α-^{32}P]CTP-labeling experiments [14].

Our failure to obtain evidence for 5′-extended tRNA precursors in mitochondrial RNA from *L. tarentolae* and *T. brucei* does not, of course, eliminate the possibility that such precursors exist and are undetected by the experimental methods employed. However, our results are consistent with previous in vivo transfection data obtained with both *L. tarentolae* [10] and *T. brucei* [11,13], in which mitochondrial targeting of episomally-expressed tRNAs was independent of the presence of a 5′-flanking genomic sequence in the tRNA gene. We conclude that the hypothesis that tRNAs are imported into mitochondria of kinetoplastid protozoa as 5′-extended precursors is an attractive theory but, however, remains to be verified.

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