

# Modification of the universally unmodified uridine-33 in a mitochondria-imported edited tRNA and the role of the anticodon arm structure on editing efficiency

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## ABSTRACT

Editing of tRNA has a wide phylogenetic distribution among eukaryotes and in some cases serves to expand the decoding capacity of the target tRNA. We previously described C-to-U editing of the wobble position of the imported tRNA<sup>Trp</sup> in *Leishmania* mitochondria, which is essential for decoding UGA codons as tryptophan. Here we show the complete set of nucleotide modifications in the anticodon arm of the mitochondrial and cytosolic tRNA<sup>Trp</sup> as determined by electrospray ionization mass spectrometry. This analysis revealed extensive mitochondria-specific post-transcriptional modifications, including the first example of thiolation of U33, the “universally unmodified” uridine. In light of the known rigidity imparted on sugar conformation by thiolation, our discovery of a thiolated U33 suggests that conformational flexibility is not a universal feature of the anticodon structural signature. In addition, the *in vivo* analysis of tRNA<sup>Trp</sup> variants presented shows a single base-pair reversal in the anticodon stem of tRNA<sup>Trp</sup> is sufficient to abrogate editing *in vivo*, indicating that subtle changes in anticodon structure can have drastic effects on editing efficiency.

**Keywords:** editing; import; mitochondria; modification; thiolation; tRNA; U33

## INTRODUCTION

Nucleoside modifications are a universal feature of tRNAs, necessary for fine-tuning the tRNA structure for translational efficiency and in a few cases modifications even change the tRNA amino acid specificity (Muramatsu et al., 1988a, 1988b; Weber et al., 1990). Although in various organisms specific modifications at certain positions in a particular tRNA show phylogenetic conservation, only a few positions and/or modifications are highly conserved (Auffinger & Westhof, 1998, 1999). These include the 3' CCA, pseudouridine

( $\Psi$ ) at position 55, and the unmodified pyrimidine at position 33 (uridine in 97% of the cases; Sprinzl et al., 1998a).

Unlike RNA modification, editing of tRNAs has been observed in several eukaryotes ranging from protists to metazoans (Lonergan & Gray, 1993; Yokobori & Pääbo, 1995, 1997; Marechal-Drouard et al., 1996; Antes et al., 1998; Price & Gray, 1999; Lavrov et al., 2000). RNA editing typically restores base pairing at various positions in the tRNA molecule, regenerating conserved structural motifs important for tRNA processing and function. In cases where tRNA editing alters the anticodon sequence, enzymatic deamination of adenosine to inosine is the common mechanism and it helps expand the base-pairing ability of the tRNA codon–anticodon interaction (Auxilien et al., 1996; Gerber & Keller, 1999). Two instances of cytidine (C)-to-uridine (U) alterations of the anticodon sequences of tRNAs have also been reported. In marsupials, a single C-to-U

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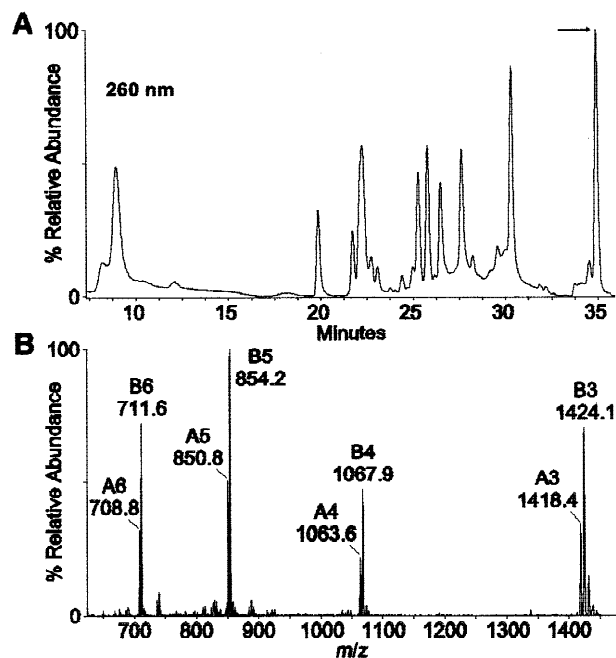
editing event in the second position of the anticodon changes the decoding properties of a mitochondria-encoded tRNA<sup>ASP</sup> (Janke & Pääbo, 1993), and in *Leishmania tarentolae*, the single nucleus-encoded tRNA<sup>TRP</sup> is imported into the mitochondrion and undergoes C-to-U editing at the wobble position, thereby allowing decoding of UGA codons as tryptophan (Alfonzo et al., 1999).

In many organisms, a set of nucleus-encoded tRNAs is imported into the mitochondria (Schneider & Marechal-Drouard, 2000). The number of imported tRNAs varies from a single tRNA in yeast (Kolesnikova et al., 2000) to all mitochondrial tRNAs in trypanosomatids (Simpson et al., 1989; Hancock & Hajduk, 1990). Little is known about the extent, nature, or function of nucleotide modifications of such imported tRNAs. Modification of U33 in mitochondrial (mt) tRNA<sup>Tyr</sup> and C32 in mt tRNA<sup>Lys</sup> and tRNA<sup>Leu</sup> in *Trypanosoma brucei* has been reported, but the nature of these modifications was not established (Schneider et al., 1994). In this study, the imported tRNA<sup>TRP</sup> from *L. tarentolae* was investigated using newly developed suites of methods based on electrospray ionization mass spectrometry (ESI-MS). We show that, after mitochondrial import, tRNA<sup>TRP</sup> is the substrate for multiple mitochondria-specific modifications, including an unprecedented thiolation at the C2 position of U33. A second modification affects the edited position (the wobble nucleotide, C34) and suggests the presence of either a novel editing activity or a novel editing regulatory mechanism in this organism. We also show that a single base-pair reversal in the anticodon stem of tRNA<sup>TRP</sup> is sufficient to abrogate editing in vivo, indicating that subtle changes in anticodon structure can have drastic effects on editing efficiency.

## RESULTS

### The anticodon arm of mt tRNA<sup>TRP</sup> is extensively modified

A portion of the purified mt tRNA<sup>TRP</sup> was digested to completion with RNase T1, and the hydrolysate was analyzed using directly combined liquid chromatography/ESI-MS (LC/ESI-MS; Felden et al., 1998; Fig. 1). Relative molecular masses ( $M_r$ ) for each oligonucleotide were determined and compared with masses of unmodified oligonucleotides calculated from the gene sequence (Kowalak et al., 1993). The edited U34 is predicted to occur in a 13-mer consisting of a portion of the anticodon stem and the entire anticodon loop (ASL). The ASL is the longest T1-derived oligonucleotide and was assigned as the component (s) eluting at 34.9 min (Fig. 1A), based on mass measurement. The electrospray ionization mass spectrum indicates that two components are present in this peak in an ~2:1 ratio (Fig. 1B): a minor ASL (component A;  $M_r$  4258.7, av-



**FIGURE 1.** LC/ESI-MS of RNase T1-digested tRNA<sup>TRP</sup> from *L. tarentolae* mitochondria. **A:** HPLC separation of products from a total RNase T1 digest of tRNA<sup>TRP</sup> (detection at 260 nm). **B:** ESI mass spectrum of the 34.9-min peak in **A** showing two components, A and B. The accompanying numerals indicate the number of charges ( $z$ ), for example, B5 is  $(M_B-5H)^{5-}$ . The arrow in **A** denotes the position of the peak eluting at 34.9 min.

erage mass scale) and a major ASL (B;  $M_r$  4275.8), with the more abundant species being 17 Da greater in mass than the minor one. We conclude that this mass difference reflects a C-to-U edit (+1 Da) and presence of an additional modification accounting for 16 Da (+O, or +S - O). Compared with the calculated mass of the unmodified T1-derived ASL, both of the modified 13-mers contain modifications accounting for an additional 110 Da.

A census of modified nucleosides in the full-length tRNA<sup>TRP</sup> was obtained from LC/ESI-MS of a total nuclease P1 digest (Crain, 1990; Pomerantz & McCloskey, 1990; Fig. S1<sup>7</sup>). The following modified nucleosides were identified: dihydrouridine (D), pseudouridine ( $\Psi$ ), 3-amino-3-carboxypropyluridine (acp<sup>3</sup>U), 2'-O-methylpseudouridine ( $\Psi^m$ ), 2'-O-methylcytidine (Cm), 5-methyluridine (m<sup>5</sup>U), 7-methylguanosine (m<sup>7</sup>G), 2-thiouridine (s<sup>2</sup>U), 2'-O-methyluridine (Um), 2'-O-methylguanosine (Gm), N<sup>2</sup>-methylguanosine (m<sup>2</sup>G), and N<sup>6</sup>-isopentenyladenosine (i<sup>6</sup>A). Given knowledge of the modified nucleoside content, the data from analysis of the total RNase T1 digest (Fig. 1) were retrospectively interrogated to reconstruct base fragment (Crain et al., 1999) ion chromatograms for eligible mod-

<sup>7</sup>Supplementary material (Figures S1–S6 and Tables S1 and S2) can be found at the following web site: <http://www.rna.ucla.edu/suppl/index.html>.

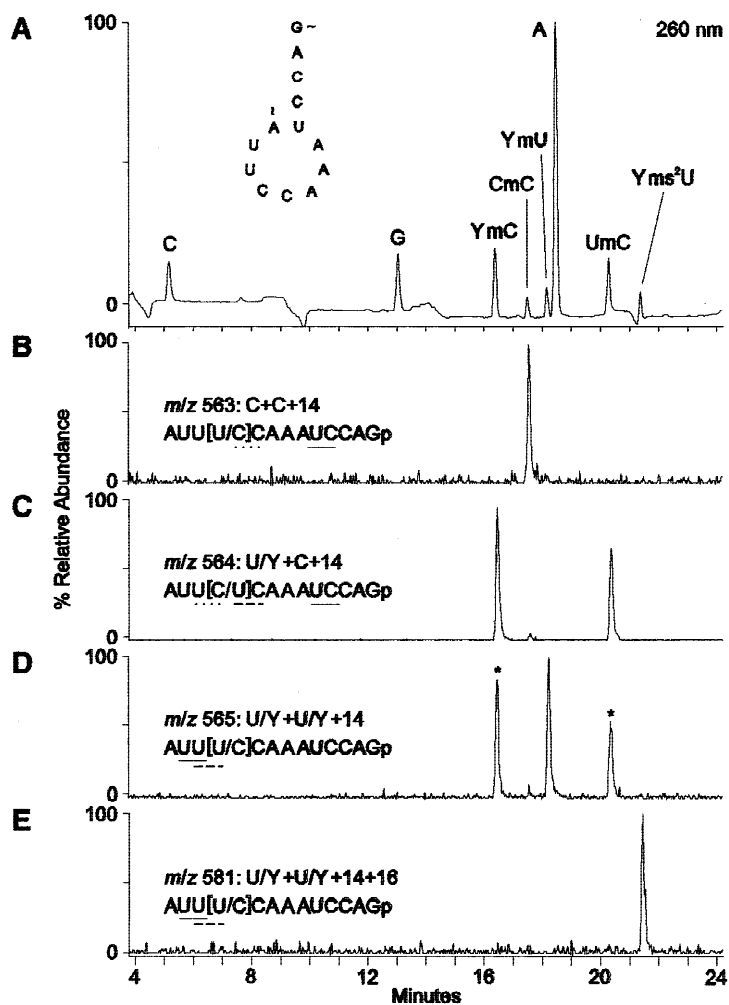
ified nucleosides (Fig. S2). When correlated with the oligonucleotide molecular mass data, this approach allows assignment of specific modifications to the given T1 oligonucleotides that contain them (Crain et al., 1999); sequence assignments of the RNase T1-derived oligonucleotides are given in Table S1 and Figure S3. Based on presence of  $s^2U$  (Fig. S1), chromatographic correspondence of  $m/z$  127 ( $B^-$  for 2-thiouridine) and the 34.9 min peak (Fig. S2),  $s^2U$  is assigned to the edited ASL. The edit and thiolation thus account for the 17-Da mass difference between the two ASLs.  $N^6$ -isopentenyladenosine is also present, accounting for 68 Da of the 110-Da mass increment present in both ASLs, relative to the unmodified RNA predicted from the gene sequence. As no other base-modified nucleosides are apparent in the ASL 13-mers (Fig. S2; D,  $acp^3U$ ,  $m^5U$ ,  $m^2G$ , and  $m^7G$  would be eligible candidates based on the total census of modifications, Fig. S1), the remaining 42 Da were attributed to the presence of three ribose-methylated nucleosides ( $\Psi m$ , Cm, and Um are eligible candidates).

In a separate analysis, the 13-mer ASL fractions derived from RNase T1 digestion of cytoplasmic (ct)

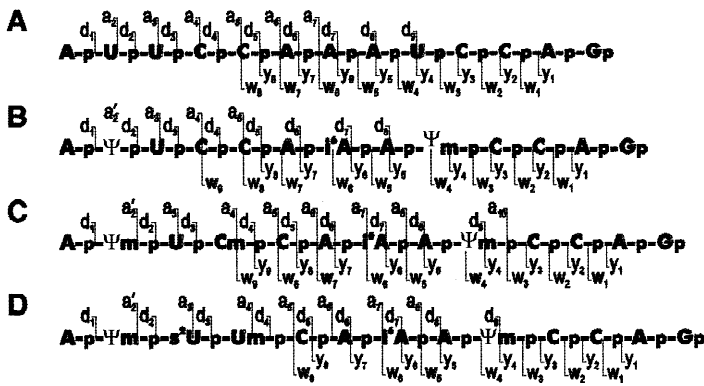
and mt  $tRNA^{Trp}$  were gel purified, and the mixture of edited and unedited mt ASLs (which could not be separated) was totally digested with RNase T2 and alkaline phosphatase. The digestion products should contain alkali-stable dinucleotides (NmM) whose 5' members represent the three Nm residues proposed to account for the unassigned 42-Da mass increment (see above), and ideally, permit their sequence placement in the ASLs. The LC/ESI-MS analysis (Fig. 2 and Figs. S4 and S5) revealed the presence of CmC,  $\Psi mC$ , UmC,  $\Psi mU$  (or UmU), and a dinucleotide assigned as  $\Psi ms^2U$  based on the product ion mass spectrum (Phillips & McCloskey, 1993; Fig. S4), relative retention times, and the presence of  $s^2U$  (but not  $s^4U$  or  $s^2Um$ ; Fig. S6). Because there are only two contiguous uridines in the ASL 13-mers (32-UU-33 in both, and 33-UU-34 in the edited tRNA), we conclude that U33 must be modified.

### Uridine-33 is thiolated in the mt $tRNA^{Trp}$

As multiple locations are possible for all of the RNase T2-derived dinucleotides, the sequences of ASL 13-mers from both mt  $tRNA^{Trp}$  molecules and from the ct



**FIGURE 2.** LC/ESI-MS of a total RNase T2 digest of the gel-purified RNase T1-derived anticodon 13-mer fraction of *L. tarentolae* mt  $tRNA^{Trp}$ . **A:** HPLC separation of products (UV detection at 260 nm); i<sup>6</sup>A elutes at 37.2 min (not shown). The sequence shown represents the structure of the unmodified ASL 13-mer predicted from the gene sequence. **B–E:** Reconstructed ion chromatograms from the mass spectra. **B:**  $m/z$  564  $MH^+$  of (U/Ψ)mC. **C:**  $m/z$  565,  $MH^+$  of (U/Ψ)mU. **D:**  $m/z$  563, the protonated molecular ion ( $MH^+$ ) of CmC; asterisks denote peaks representing the <sup>13</sup>C isotope peaks of (U/Ψ)mC (**B**). **E:**  $m/z$  581,  $MH^+$  of  $\Psi ms^2U$ . The underlined residues show sequence constraints dictated by the data in **B–E**.

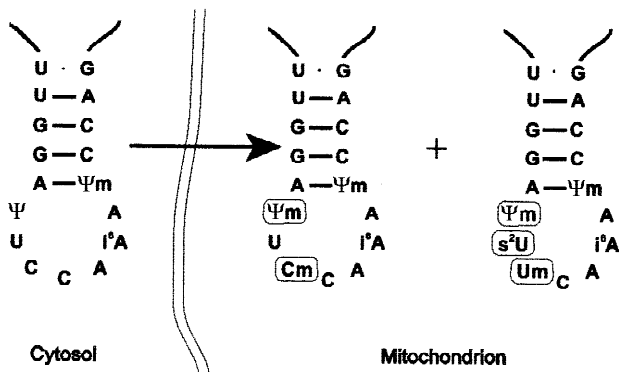


**FIGURE 3.** Sequence-determining ions observed in product ion mass spectra from collision-induced dissociation of *L. tarentolae* tRNA<sup>Trp</sup> RNase T1-derived anticodon ASL13-mers. **A:** Synthetic unmodified RNA. **B:** Cytoplasmic tRNA<sup>Trp</sup>. **C:** Minor mitochondrial tRNA<sup>Trp</sup>. **D:** Major mitochondrial tRNA<sup>Trp</sup>. Ion type designations (McLuckey et al., 1992) have been modified here for simplicity: for example, d<sub>1</sub> is d<sub>1</sub>-H<sub>2</sub>O and a<sub>2</sub> is a<sub>2</sub>-B<sub>2</sub> (the base at position 2); w and y ions are as originally described. Sequence data are presented in Table S2. The conventional tRNA numbering has been used throughout.

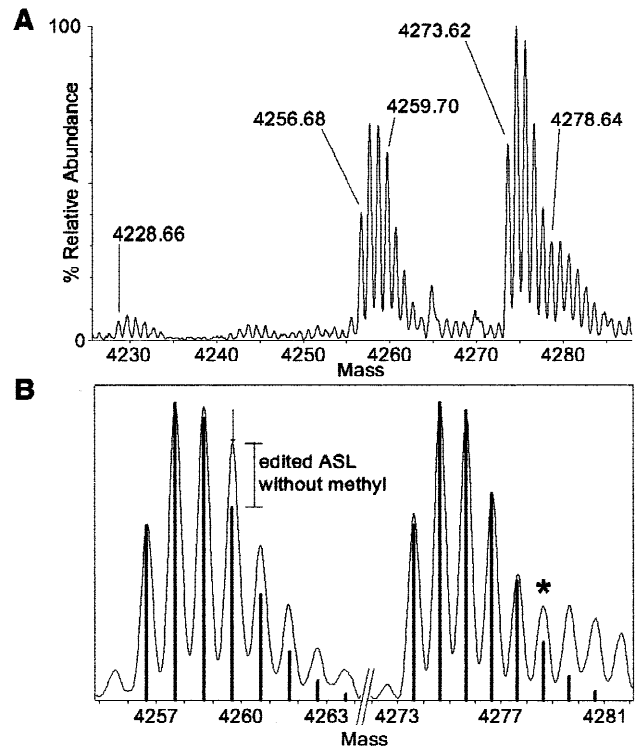
precursor tRNA<sup>Trp</sup> were determined ab initio using tandem mass spectrometry (Ni et al., 1996). The two mt species could be independently sequenced by tandem mass spectrometry (MS/MS) because of their different molecular mass values, despite their occurrence in an unresolved mixture. The resulting sequences (Fig. 3 and Table S2) reconcile the measurements of molecular mass, modified nucleoside identities, and dinucleotide sequences, and lead to several remarkable and unexpected conclusions. First, 2-thiouridine, previously known to occur only at anticodon position 34, is present instead at position 33 in the edited ASL (Fig. 3D), a site for which there is no precedent for modification in any of the 550 published tRNA sequences (Sprinzl et al., 1998). Second, as shown in Figure 4, the amount of postimportation alteration of the ASL in the mitochondria is exceptional: methylation of Ψ32 (Fig. 3C, D) and C34 (Fig. 3C), editing of C(m)34 to U(m)34 (Fig. 3D), and thiolation of U33 (Fig. 3D).

In conjunction with the sequencing analyses, high-resolution mass measurements were made on the ct tRNA<sup>Trp</sup> ASL and the mixture of unedited and edited mt tRNA<sup>Trp</sup> ASLs. The calculated *M<sub>r</sub>* values for the three ASLs are 4228.635, 4256.667, and 4273.628. The mea-

sured mass values in Figure 5A agree with these calculated values to within 0.02 Da or better, further supporting the structures derived from the sequencing data. Moreover, the high resolving power of the quadrupole-TOF mass spectrometer permits the masses of the principal (all-light) ASLs to be resolved from those of their naturally occurring heavy isotopes (Fig. 5B) and subjected to comparison with their theoretical abundances. Presence of an additional species correspond-



**FIGURE 4.** Summary of mitochondrial editing and modification events (boxed residues) in the anticodon stem-loop region of the imported cytoplasmic tRNA<sup>Trp</sup>.



**FIGURE 5.** Molecular mass values derived from high-resolution mass measurement of gel-purified mt tRNA<sup>Trp</sup> ASLs. **A:** *M<sub>r</sub>* values determined from mass spectra of directly infused gel-purified ASL 13-mers. **B:** Superposition of the principal isotope clusters in **A**, upon the theoretical patterns (vertical bars) that show both mass and relative abundance corresponding to the oligonucleotides shown in Figures 3C, D. The asterisk denotes the Na salt of the unedited ASL (*M<sub>r</sub>* 4,256.68).



ing to a processing intermediate is strongly implied by the discordance between theoretical and measured isotope abundances for mass 4259.7 (arrow, Fig. 5B), and associated heavy isotopes. This species corresponds to an edited ASL having only two of the three methyl groups ultimately present. These findings raise immediate questions regarding the interdependence of these modification events and their roles in tRNA editing and importation.

### Changes in the anticodon arm affect editing of the mt tRNA<sup>Trp</sup>

Given the large extent of modification of the ASL of the imported mt tRNA<sup>Trp</sup>, we investigated the specific features of the tRNA<sup>Trp</sup> that the editing enzyme may utilize for recognition and discrimination in a cellular pool of different tRNAs. In *L. tarentolae*, other mitochondrial tRNAs containing a C as the wobble base, such as tRNA<sup>Leu</sup> (CAG), tRNA<sup>Val</sup> (CAC), tRNA<sup>Glu</sup> (CTC), and tRNA<sup>Gln</sup> (CTG), are not subjected to editing (data not shown). We have studied the sequence requirements for tRNA<sup>Trp</sup> editing in vivo by transfecting cells with plasmids containing mutant tRNA<sup>Trp</sup> genes. Previous work showed that *L. tarentolae* cells can express mutant tRNAs from transfected plasmids and that the tRNAs can be imported into the mitochondrion (Lima & Simpson, 1996; Sbicego et al., 1998). To create specific hybridization sites for RT-PCR primers, five nucleotide changes were introduced in the anticodon arm of the wild-type tRNA<sup>Trp</sup> sequence, generating the MASL (for mutant of the anticodon stem loop) construct (Fig. 6A). These nucleotide changes allowed amplification of transfected MASL tRNAs without interference from the endogenous wild-type tRNA<sup>Trp</sup>. This set of mutations was chosen using published sequence alignments of all tRNA<sup>Trp</sup> genes (Sprinzl et al., 1998); only positions that covary in the sequence alignment and preserve base pairing were targeted (data not shown). *L. tarentolae* cells were transfected with the pLY15 plasmid (Sbicego et al., 1998) containing the MASL tRNA gene, and the tagged tRNA<sup>Trp</sup> was amplified from mitochondrial RNA, and the resulting clones were scored for C34-to-U34 editing. As shown in Figure 6B (lanes 1 and 3), these mutations did not affect transcription or mitochondrial import of the transfected tRNA variant. The extent of editing of the MASL variant was similar to that of the wild-type tRNA<sup>Trp</sup>, as shown by the *Hinfl*-resistant RT-PCR product (Fig. 6C, lane 8, and Fig. 7, MASL 31A-39U). These data demonstrate that this MASL tRNA<sup>Trp</sup> variant is a suitable substrate for studying the effect of other anticodon arm mutations on tRNA editing in vivo.

A single base-pair reversal was introduced at the last position (or at the next to the last position) of the anticodon stem of the MASL tRNA<sup>Trp</sup> (MASL 31U-39A; Fig. 7). These mutations had no effect on transcription

of the gene or on import of the tRNA into the mitochondrion (data not shown), but completely inhibited C34 to U34 editing (Fig. 7). Several testable hypotheses for the lack of editing in the MASL 31U-39A variant include: (1) a requirement for a specific base pair at the base of the anticodon stem, such as in the formation of  $\Psi$  in the T $\Psi$ C loop of yeast tRNAs (Becker et al., 1997); (2) a specific requirement for  $\Psi$  at position 39, which was proposed to be necessary to ensure anticodon loop stability of tRNAs, either by hydrogen bonding with A31 or by the ability to stack with neighboring nucleotides (Durant & Davis, 1999); (3) a requirement for increased stability at the last position of the stem; (4) a requirement for interactions with neighboring nucleotides within the anticodon stem.

To test these hypotheses, additional tRNA<sup>Trp</sup> variants were constructed, expressed in *L. tarentolae* and scored for C34-to-U34 editing by sequencing multiple independent clones of the RT-PCR products (Fig. 7). The A-U base pair at position 31–39 was substituted by a G-C base pair (Fig. 7, MASL 31G-39C) to prevent formation of  $\Psi$  at position 39 and also to change the specific 31–39 base pair. These mutations had no effect on C34-to-U34 editing, ruling out the possibility that either  $\Psi$ 39 or a specific A-U base pair was required for efficient editing.

The A-U base pair at 31–39 was then replaced with a more stable C-G base pair (Fig. 7, MASL 31C-39G). These mutations completely inhibited C34-to-U34 editing, indicating that an increased relative stability of the 31–39 base pair is not sufficient to ensure efficient anticodon editing. Furthermore, replacement of the G-C pair at position 30–40 with a C-G pair (Fig. 7, MASL 30C-40G), potentially disrupting the purine–purine stacking of A31 and G30, had no effect on C34-to-U34 editing, suggesting that interactions between the bases within the anticodon stem have minor contributions, if any, to editing efficiency. We conclude that the inhibitory effect of the MASL 31U-39A (Fig. 7) and MASL 31C-39G (Fig. 7) mutations on editing may reflect the role this position plays in anticodon loop structure, either by directly affecting the structure or by affecting the formation of specific modified nucleotides necessary for anticodon loop recognition by the editing enzyme.

### Editing is not required for thiolation

The discovery of a novel s<sup>2</sup>U33 nucleotide modification in the imported mt tRNA<sup>Trp</sup> but not in the ct tRNA<sup>Trp</sup> raised the possibility of a relationship between this modification and editing at C34. Because this represents the sole sulfur modification in this tRNA (Figs. S1, S2, and S6 and Table S1), the extent of U33 thiolation in the mitochondrial tRNA<sup>Trp</sup> could be assayed by separating the sulfur-containing tRNA<sup>Trp</sup> from non-thiolated species (Emilsson et al., 1992) on APM (*N*-



	MASL 31A-39U	MASL 31U-39A	MASL 31G-39C	MASL 31C-39G	MASL 30C-40G
Edited:	13	0	11	0	13
Non-edited:	17	30	19	30	17
% edited:	43	0	37	0	43

**FIGURE 7.** Effect of single base-pair changes on editing in vivo. Only the stem-loop portions of the different tRNA variants are shown. Names above each construct represent the actual position and type of change introduced in the tRNA sequences; numbering is according to the Sprinzl convention (Sprinzl et al., 1998). Boldface letters represent the nucleotide changes introduced. After RT-PCR, cloning, and sequencing of the different variants, individual clones were scored for whether or not they were edited in vivo. Thirty independent clones were sequenced. The percentage of edited clones was calculated by dividing the number of edited clones by the sum of edited and non-edited clones times 100.

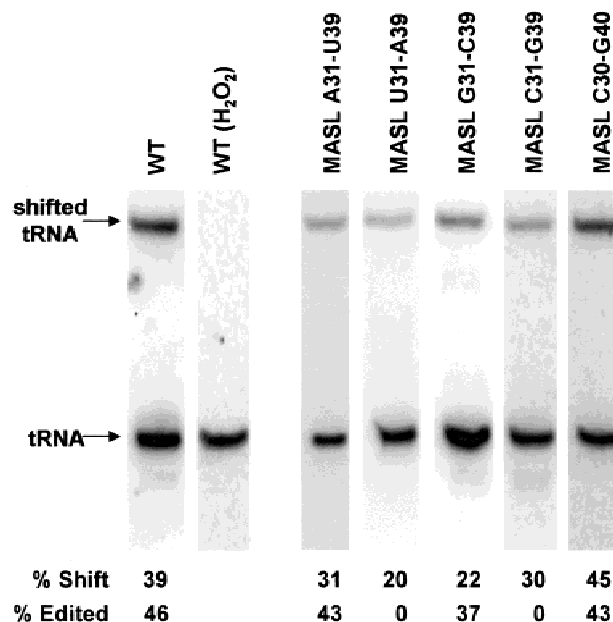
plays in C34-to-U34 editing thus remains an open question.

## DISCUSSION

### The anticodon structural signature

One of the most conserved structural features in tRNA is a characteristic U-turn in the anticodon loop (Auffinger & Westhof, 2001). This structure is maintained by subtle interactions involving hydrogen bonding, changes in sugar conformation, and base stacking between several nucleotides in the anticodon stem loop. The interplay of structural conservation and structural variation in the anticodon stem loop is a corollary of the extended anticodon hypothesis of Yarus (1982), in which nucleotides in the upper loop and the stem expand the information content of the three anticodon nucleotides and affect the overall translational efficiency of a given tRNA.

Two models based on structural and phylogenetic analyses have been proposed for the stabilization of the U-turn structure in tRNAs. One proposal states that a dynamic sugar pucker is essential for the formation of the universal anticodon structure (Ashraf et al., 1999a). This assumes that position U33 of the anticodon is universally unmodified. A second model maintains that formation of a bifurcated hydrogen bond between the (U33)O2' and the NH<sub>2</sub> and C5-H groups of C35 are more important than a dynamic sugar pucker (Auffinger & Westhof, 2001). Our finding that U33 is thiolated in the imported mitochondrial tRNA<sup>Trp</sup> of *L. tarentolae* leads to the conclusion that, whereas conformational flexibility about the sugar might be impor-



**FIGURE 8.** Editing is not required for thiolation in vivo. Equal amounts of mitochondrial RNA from the different variants shown in Figure 7 were electrophoretically separated on APM gels and analyzed by northern hybridization as described in Materials and Methods. Shifted bands represent the thiolated species. Lane 1: Wild-type mitochondrial RNA from untransformed cells. Lane 2: The same RNA as in lane 1 but treated with hydrogen peroxide prior to loading on the gel for use as a control (see Materials and Methods). Lane 3: The tRNA<sup>Trp</sup> variant from Figure 6 used to study editing in vivo. Lanes 4–7, the variants MASL 31A-39U, 31U-39A, 31G-39C, 31C-39G, and 30C-40G as in Figure 7.

tant to maintain a desirable anticodon structure for many tRNAs, this fact is not a universal feature of the anticodon structural signature. In view of the known rigidity imparted on the sugar conformation by the presence of 2-thiouridine (Kumar & Davis, 1997; Ashraf et al., 1999b), we speculate that at least in the case of tRNA<sup>Trp</sup>, s<sup>2</sup>U33 should strongly favor a C3'-endo sugar conformation.

### A novel tRNA editing activity or novel editing regulation?

The ASL population analyzed (Fig. 5B) represents a steady-state distribution of tRNAs and accordingly does not permit determination of the order of nucleotide modification. If we assume that ribose methylation of C34 occurs before editing, this would imply that there is a novel editing activity with specificity for a modified nucleotide. Conversely, if we assume methylation occurs after editing of C34 to U34 and the editing enzyme requires an unmethylated C34 as substrate, this may provide a novel mechanism to regulate editing by nucleotide modification, thus ensuring that not all of the imported tRNA<sup>Trp</sup> is edited as was observed in Figure 7. The presence of the two tryptophan tRNAs in the mitochondrion is most likely required to decode the two tryptophan codons (UGA and UGG) in mitochondrial genes.

We also propose that base stacking between a purine at position 31 and nucleotides in the 5' side of the anticodon loop may be used by the editing enzyme for efficient recognition. This proposal takes into consideration the presence of  $\Psi$ m at position 32 of tRNA<sup>Trp</sup> and its tendency to increase stacking of neighboring 5' purines (Davis, 1995). The presence of a purine at position 31 together with the modifications at positions 32 through 34 should impart structural rigidity to the anticodon loop as a whole, including the U-turn.

Alternatively, the editing enzyme may require flexibility on the 3' side of the loop (36–39) induced by a pyrimidine at position 39, as purines with their tendency to stack could make the 3' side of the anticodon loop too rigid for recognition. This could explain why a C at position 39 works as well as the U in the natural substrate. This explanation, however, is less likely, due to the presence of  $\Psi$ m at position 39 in the natural tRNA<sup>Trp</sup>. The combination of stacking tendency by  $\Psi$  with the tendency towards 3'-endo puckering of the methylated ribose should already make the 3' side of the loop rigid, thus ruling out an absolute requirement for flexibility.

This work has highlighted the utility of electrospray ionization mass spectrometry as a way to elucidate the primary structure of naturally modified nucleic acids. This approach has unveiled a remarkable extent of posttranscriptional modifications of the mitochondria-imported edited tRNA<sup>Trp</sup> of *L. tarentolae*. This is the most extensively modified tRNA anticodon stem-loop described so far and is the first example of a coupling of modification to editing, which raises the question as to whether this degree of modification occurs with other imported tRNAs. Schneider et al. (1994), on the basis of several undefined nucleotide modifications in *T. brucei*, have suggested that nucleotide modification of mt imported tRNAs is required for intramitochondrial functions such as translation. We would like to expand on this idea and to propose a general "tRNA re-adaptation" hypothesis that eukaryotes may have evolved the use of nucleotide modifications of mt imported tRNAs to structurally re-adapt cytosolic tRNAs to be functional in the bacteria-like translation system of the mitochondrion. Additional studies of cytosolic and imported mitochondrial tRNAs will be required to test this hypothesis. Part of this re-adaptation in the case of the tRNA<sup>Trp</sup> includes anticodon editing as well as thiolation at an unprecedented position (U33). The existence of a functional tRNA with this thiolation also suggests that conformational flexibility about the sugar is not a universal requirement for proper tRNA function.

## MATERIALS AND METHODS

### Mitochondrial and cytosol isolation

Mitochondria were isolated by the standard hypotonic lysis and Renografin density method from *L. tarentolae* cells grown

in brain-heart-infusion medium (Difco) with 10  $\mu$ g/mL hemin at 27 °C to a cell density of  $1 \times 10^8$  cells/mL (Alfonzo et al., 1999). The isolated mitochondrial fraction was treated with micrococcal nuclease (100 U/mg mitochondria, wet weight) to remove any RNA nonspecifically bound to the outside of the vesicles. This method yields mitochondria with no contamination with cytosolic RNA detectable by northern analysis (Kapushoc et al., 2000). In addition, control RT-PCR reactions for the MASL tRNAs with the supernatant from the nuclease-treated mitochondria showed no detectable products. Cytosolic RNA (together with nuclear RNA) was isolated by rupturing cells in 0.6 M sorbitol and clarification at  $22,000 \times g$  for 60 min. The RNA was isolated from the supernatant as previously described (Alfonzo et al., 1999). This method yields cytosolic RNA with less than 0.1% contamination with mitochondrial RNA (Kapushoc et al., 2000).

### RNA purification and synthesis

Native tRNA<sup>Trp</sup> (cytosolic or mitochondrial) was purified from 500 L of *L. tarentolae* culture by affinity purification as previously described (Alfonzo et al., 1999). Approximately 666.6 g of highly purified mitochondria (wet weight) were used for the isolation of mitochondrial RNA. The unmodified RNA 13-mer was synthesized at the University of Utah DNA and Peptide Core facility, and used for instrument tuning and as a sequence model (Fig. 3A).

### Mass spectrometry

Total digestion of tRNA<sup>Trp</sup> with RNase T1 and analysis of the products by negative-ion LC/ESI-MS (Figs. 1 and S2) was conducted essentially as described (Felden et al., 1998), except for the addition of a second scan function at elevated cone voltage (65 V vs. 37 V) to release modified bases (Crain et al., 1999). tRNA<sup>Trp</sup> and its gel-purified RNase T1-derived 13-mer fraction were digested to nucleosides with nuclease P1, phosphodiesterase I, and bacterial alkaline phosphatase (BAP; Crain, 1990) and analyzed by positive ion LC/ESI-MS (Felden et al., 1998; Figs. S1 and S3). The gel-purified 13-mer fraction was totally digested with RNase T2 (Sigma R6398) for 1 h at 37 °C (1 U/20 pmol RNA); 0.6 U BAP was added and the incubation continued for 1 h (Fig. 2). All of these LC/MS studies were conducted using a Hewlett-Packard (Palo Alto, California) 1090 DAD liquid chromatograph interfaced to a Micromass (Beverly, Massachusetts) Quattro II triple quadrupole mass spectrometer with MassLynx v. 3.2 instrument control and analysis data system.

High-resolution mass measurements (Fig. 5) and sequencing of unmodified (synthetic) ct and mt tRNA<sup>Trp</sup> 13-mers (Fig. 3) and selected RNase T2-derived dinucleotides were conducted with a Micromass Q-ToF 2 tandem mass spectrometer, using ESI with negative ions detected. Sample introduction for mass measurements and CID analyses of gel-purified 13-mers was by loop injection with the sample dissolved in MeOH + H<sub>2</sub>O, 1:1. The -4 charge state molecular ions were mass selected for dissociation (collision energy 30 eV Elab for all 13-mers) to derive sequences (Fig. 3).

A Waters (Milford, Massachusetts) Cap LC liquid chromatograph was interfaced to the Q-ToF 2 for LC/MS/MS analysis of the RNase T2-derived dinucleotides  $\Psi$ mp-s<sup>2</sup>Up (from the



edited ASL) and  $\Psi$ mpCp (ct ASL; Figs. S4 and S5). The HPLC eluents were the 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/TEA/MeOH solvents, prepared as described (Apffel et al., 1997), except that the A and B buffers were 0.2 M HFIP. The gradient was developed using curve 6 as follows: 0–5 min, 100% A; 40 min, 75% A; 50 min, 0% A; 50–55 min, 0% A. The column (Phenomenex Luna C18; 150  $\times$  0.5 mm) was eluted at a flow rate of 6  $\mu$ L/min. For these and the LC/MS analyses using the Quattro II instrument, all digests were injected directly onto the columns, without prior clean-up, and all eluents were directed into the mass spectrometer (s) without splitting.

### Thiolation assays

Total mitochondrial RNA was electrophoretically separated on 7 M urea/8% acrylamide gels cast with 50  $\mu$ M APM. The samples were denatured by heating at 95  $^{\circ}$ C for 5 min under denaturing conditions (7 M urea). Equal amounts of RNA were loaded per lane (2.5  $\mu$ g). After electrophoresis, the gels were soaked in TBE buffer containing 200 mM  $\beta$ -mercaptoethanol, stained with ethidium bromide for visualization under UV light, and blotted onto a Zetaprobe membrane (BioRad) for northern analysis. Northern analysis was carried out as recommended (BioRad). Control northern hybridizations were carried out with each  $^{32}$ P-labeled oligomer to show that under the hybridization conditions used, the oligomers would only recognize their intended target, thus ruling out the possibility of spurious hybridization between mutant-specific oligomers and the endogenous wild-type tRNA<sup>Trp</sup> (data not shown). The hydrogen peroxide treatment was carried out as previously described (Watanabe et al., 1994), as a control to show that the observed shift is due to thiolation of the tRNA in question.

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