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C to U editing of the anticodon of imported mitochondrial tRNA^{Trp} allows decoding of the UGA stop codon in *Leishmania tarentolae*

Juan D.Alfonzo[1], Valerie Blanc[1], Antonio M.Estévez[1], Mary Anne T.Rubio[2] and Larry Simpson[1,2,3,4]

[1]Howard Hughes Medical Institute and [3]Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095-1662 and [2]Department of Medical Microbiology, Immunology and Molecular Genetics, UCLA School of Medicine, Los Angeles, CA 90095, USA

[4]Corresponding author
e-mail: simpson@hhmi.ucla.edu

All mitochondrial tRNAs in kinetoplastid protists are encoded in the nucleus and imported into the organelle. The tRNA^{Trp}(CCA) can decode the standard UGG tryptophan codon but can not decode the mitochondrial UGA tryptophan codon. We show that the mitochondrial tRNA^{Trp} undergoes a specific C to U nucleotide modification in the first position of the anticodon, which allows decoding of mitochondrial UGA codons as tryptophan. Functional evidence for the absence of a UGA suppressor tRNA in the cytosol, using a reporter gene, was also obtained, which is consistent with a mitochondrial localization of this editing event. *Leishmania* cells have dealt with the problem of a lack of expression within the organelle of this non-universal tRNA by compartmentalizing an editing activity that modifies the anticodon of the imported tRNA.

Keywords: anticodon/editing/mitochondria/translation/tRNA

Introduction

Several types of tRNA editing have been described previously (Janke and Paabo, 1993; Antes *et al*., 1998; Price and Gray, 1998). Nucleotide substitution/modification editing of mitochondrial tRNAs that restores base pairing at conserved sites in the acceptor stem occurs in *Acanthamoeba* spp. (Lonergan and Gray, 1993), *Chytridiomycete* fungi and some animals (Price and Gray, 1998). In marsupials, a single nucleotide modification in the second position of the anticodon restores the decoding properties of a mitochondrial-encoded tRNA^{Asp} (Janke and Paabo, 1993). In *Physarum polycephalum*, several mitochondrial-encoded tRNAs show both cytidine (C) insertion and U insertion editing (Miller *et al*., 1993). In addition, specific deamination of adenosine to inosine in tRNAs occurs in *Saccharomyces cerevisiae* (Grosjean *et al*., 1996; Gerber *et al*., 1998)

The mitochondrial genome of trypanosomatid protozoa consists of 20–50 catenated maxicircle molecules containing two rRNA genes and 18 protein-coding genes (Simpson, 1987). Approximately 12 of the protein-coding genes (depending on the species) are cryptogenes, the transcripts of which are edited to varying extents by insertion and deletion of uridines (Sollner-Webb, 1996; Alfonzo *et al*., 1997; Hajduk, 1997; Kable *et al*., 1997; Sloof and Benne, 1997). In addition, the mitochondrial genome contains 10 000–20 000 catenated minicircle molecules encoding the guide RNAs involved in mediating the editing process (Blum and Simpson, 1990; Pollard *et al*., 1990). No tRNAs are encoded in either the maxicircle or minicircle DNAs (Simpson *et al*., 1989; Hancock and Hajduk, 1990). There is both *in vivo* and *in vitro* evidence for mitochondrial importation of nucleus-encoded tRNAs in *Leishmania tarentolae* and *Trypanosoma brucei* (Simpson *et al*., 1989; Schneider, 1994; Lima and Simpson, 1996; Mahapatra and Adhya, 1996).

As is the case in most eukaryotes (with the exception of plants and several protists) (Gray *et al*., 1998), the mitochondrial genetic code of *L.tarentolae* deviates from the universal code in that the UGA stop codon is used as a codon for tryptophan (de la Cruz *et al*., 1984). However, in *L.tarentolae*, the single nucleus-encoded tRNA^{Trp} has a CCA anticodon that can decode the UGG tryptophan codon used in nuclear genes but can not decode the UGA tryptophan codon used in mitochondrial genes (Shi *et al*., 1994). We show here that the imported mitochondrial tRNA^{Trp} is edited at the first anticodon position to generate a UCA anticodon that allows decoding of the UGA codon as tryptophan.

Results

The imported nucleus-encoded tRNA^{Trp}(CCA) can not decode the mitochondrial UGA codon as tryptophan

In *L.tarentolae*, tRNA^{Trp} is encoded in a single-copy nuclear gene and has the anticodon CCA (Shi *et al*., 1994). This tRNA was detected previously by Northern analysis in both the cytosolic and mitochondrial compartments of this organism (Shi *et al*., 1994). However, an examination of the 18 mitochondrial maxicircle structural genes presented in Table I shows that 88% of the inferred conserved tryptophan codons are UGA, which can not be decoded by a CCA anticodon. In addition, UGA is not employed as a stop codon in any maxicircle gene.

A single C to U editing event in the first position of the anticodon of tRNA^{Trp} allows decoding of the UGA codon as tryptophan

The apparent paradox involving the decoding of UGA codons could be solved if the CCA anticodon of the mitochondrial tRNA^{Trp} is converted to a UCA anticodon by a single C to U editing event at the first anticodon position (C34) (Figure 1). C to U deamination editing occurs in several other organisms, including marsupials,

Table I. Tryptophan codon usage in *L.tarentolae* mitochondria

Gene	TRP			Stop	
	UGA	UGG	Total	UAA	UAG
ND8	1	0	1		X
ND9	2	1	3		X
MURF5	1	1	2	X	
ND7	3	0	3	X	
COIII	8	0	8	X	
Cyb	15	1	16	X	
MURF4	2	0	2	X	
MURF1	4	1	5	X	
G3	0	1	1	X	
ND1	3	0	3	X	
COII	7	0	7		X
MURF2	3	0	3	X	
COI	13	1	14	X	
G4	1	2	3	X	
ND4	8	1	9		X
G5	2	2	4	X	
RPS12	1	0	1	X	
ND5	15	1	16	X	
Total	89	12	101	14	4

The number of codons in which UGA or UGG encode tryptophan in the deduced amino acid sequences of all 18 maxicircle mitochondrial genes are shown. X indicates usage of UAA or UAG as a stop codon in each gene. ND1, 4, 5, 7, 8 and 9, NADH dehydrogenase 1, 4, 5, 7, 8 and 9; MURF1, 2, 4 and 5, maxicircle unidentified reading frame 1, 2, 4 and 5; Cyb, cytochrome *b*; COI, II and III, cytochrome oxidase subunit I, II and III; G3, 4 and 5, G-rich region 3, 4 and 5; RPS12, ribosomal protein S12.

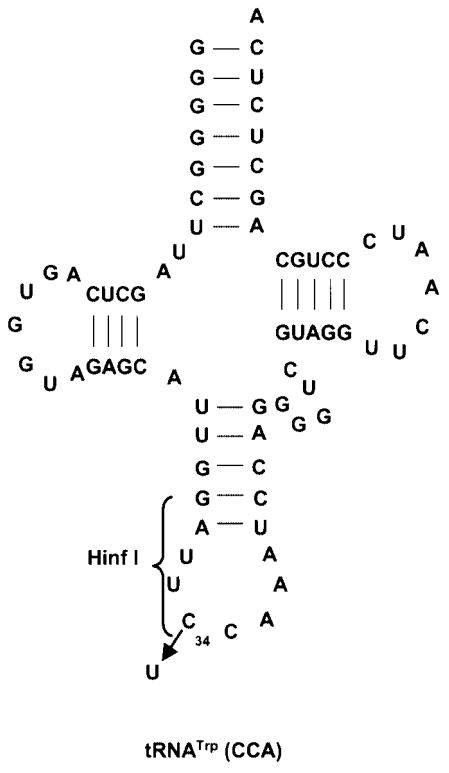


Fig. 1. Predicted secondary structure of the tRNA^{Trp}(CCA). The arrow denotes the proposed C to U editing of the first position of the anticodon to create the tRNA^{Trp}(UCA). The sequence enclosed by the bracket corresponds to the *Hin*fI site in the DNA copies of the pre-edited tRNA, which was used as a diagnostic for editing of C34. Note that the canonical nucleotide numbering system is used, although C34 actually corresponds to C33 in this tRNA.

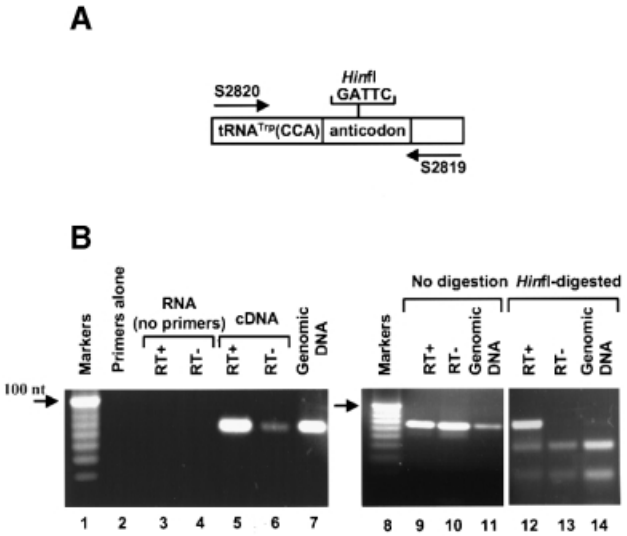


Fig. 2. RT–PCR assay for edited tRNA^{Trp} (UCA). (**A**) Diagram of RT–PCR amplification of the tRNA^{Trp}, with the *Hin*fI site in the anticodon indicated. S2820 and S2819 are the RT–PCR primers. (**B**) Ethidium bromide-stained agarose gel of the RT–PCR products of total cell RNA or genomic DNA. A 10 bp DNA ladder (Gibco-BRL) was used as size marker. In the left panel are control PCRs in which either the template (lane 2) or the primers (lanes 3 and 4) were omitted. A 70 nucleotide band was observed in both the RT–PCR lane (RT+) (lane 5) and the genomic DNA lane (lane 7). A faint 70 nucleotide band reproducibly appeared in a control reaction in which reverse transcriptase was left out during the reverse transcription step (RT–) (lane 6), which is probably due to DNA contamination of the total cell RNA sample. The right panel shows the results of digesting the PCR products with *Hin*fI. No attempt was made to do quantitative RT–PCR and therefore the relative extents of edited versus unedited RNA can not be deduced from these data. Numbers at the bottom of each panel indicate lane numbers.

plant mitochondria, chloroplasts and mammalian apoB mRNA (Smith *et al*., 1997).

An oligonucleotide (S2819) complementary to the 3′ end of tRNA^{Trp} was used as a primer to reverse transcribe total cellular RNA, and this cDNA was then PCR-amplified (RT–PCR) using the S2819 primer and the S2820 primer, which is specific for the 5′ end of this tRNA. As shown in Figure 2A, if C34 in the anticodon is modified to a U in a post-transcriptional editing event, this should destroy a unique *Hin*fI restriction site, giving rise to a *Hin*fI-resistant PCR product. A control amplification from total cellular DNA in Figure 2B (lanes 7 and 14) failed to yield a *Hin*fI-resistant fragment, showing that an edited copy of the gene was not present in the genome. However, the RT+ RT–PCR product was resistant to cleavage with *Hin*fI, as shown in Figure 2B, lanes 5 and 12. Cloning and sequencing of both the RT+ RT–PCR *Hin*fI-resistant product (Figure 2B, lane 5) and the PCR product from total DNA (Figure 2B, lane 7) confirmed that the removal of the *Hin*fI site was due to a single apparent C to U change at the first position of the anticodon (Figure 3).

The edited tRNA^{Trp}(UCA) is localized in the mitochondrion

To determine the subcellular localization of the edited tRNA, mitochondrial RNA (kinetoplast RNA, kRNA) and cytosolic RNA (Cyt RNA) were subjected to a 'poisoned' primer extension reaction (as described in Materials and methods) using a labeled oligonucleotide primer specific

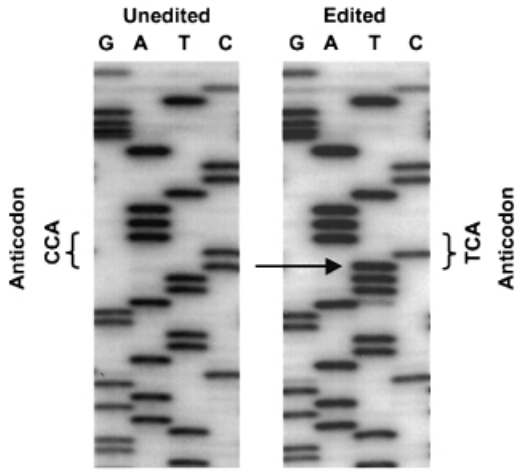


Fig. 3. Determination of the sequence of the anticodon of edited clones. The *Hin*fI-resistant RT–PCR fragment and the undigested genomic DNA PCR product from Figure 2 were cloned and sequenced. Five individual clones from both the RT+ RT–PCR reaction and the genomic DNA PCR reaction were sequenced. The left panel shows a representative sequencing ladder of a clone from the genomic DNA PCR product. The right panel shows a sequencing ladder of a clone from the RT+ RT–PCR product. A single C to T change corresponding to the first position of the anticodon (nucleotide 34) was observed only in the sequence of the RT+ RT–PCR clone, as indicated by the arrow.

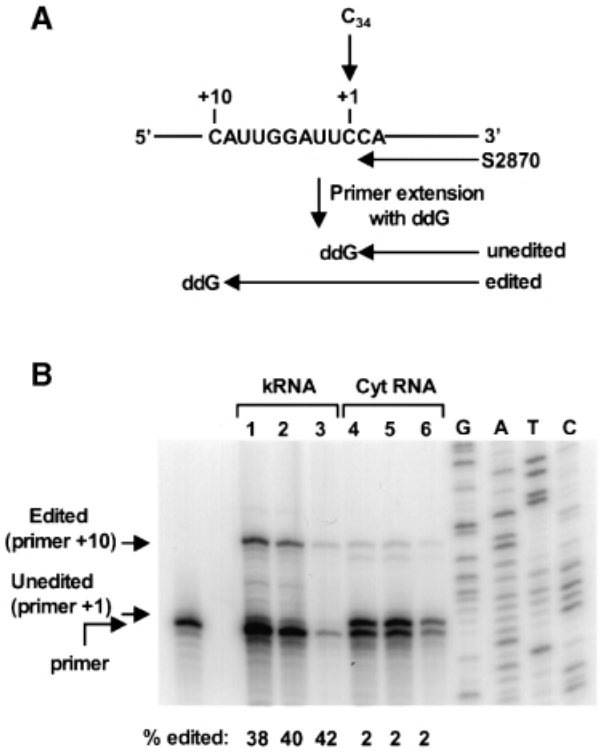


Fig. 4. Poisoned primer extension assay to detect edited and unedited $tRNA^{Trp}$ in different cell fractions. (**A**) Diagram of the extension reaction. The 5′-labeled S2870 oligonucleotide, which is specific for the $tRNA^{Trp}$, was used to extend either up to or through the editing site, yielding a primer +1 product from unedited RNA or a primer +10 product from edited RNA. The first position of the anticodon is indicated by C34 with an arrow. The numbers +1 and +10 refer to the expected products of the reaction. (**B**) Autoradiograph of a gel with the primer extension products obtained using total mitochondrial RNA (kRNA, lanes 1–3) or total cytosolic RNA (lanes 4–6). The arrows indicate the position of the products observed. To ensure saturation by the primer, either 50 (lanes 1 and 4), 25 (lanes 2 and 5) or 10 fmol (lanes 3 and 6) of primer S2870 were used per reaction. An unrelated sequencing ladder with the lanes labeled G, A, T and C was used as size marker. The relative amounts of the edited bands were measured by PhosphorImager analysis.

for the $tRNA^{Trp}$. As shown in Figure 4A, this primer (S2870) anneals just downstream and ends at nucleotide 2 of the anticodon. An unedited tRNA would yield a primer +1 extension product, and a tRNA with a C34 to U34 substitution would yield a primer +10 extension product. The results in Figure 4B show the presence of a C to U substitution at the first anticodon position in >40% of the mitochondrial $tRNA^{Trp}$ (40–70% with different mitochondrial preparations) and in <2% of the cytosolic $tRNA^{Trp}$. Furthermore, this level of edited $tRNA^{Trp}$ in the Cyt RNA fraction could be accounted for by the measured level of mitochondrial RNA contamination of the cytosolic fraction (data not shown).

Functional evidence for the absence of UGA suppressor tRNA in the cytosol

The edited $tRNA^{Trp}$(UCA) would act as a suppressor of UGA termination codons if present in the cytosol. Evidence for the absence of a detectable level of functional edited $tRNA^{Trp}$(UCA) in the cytosolic fraction was obtained by transfection of *L.tarentolae* cells with a wild-type chloramphenicol acetyltransferase (CAT) reporter gene or a mutant CAT gene in which a UGG tryptophan codon at amino acid 16 was replaced with a UGA stop codon (Figure 5A). Expression of the mutant reporter gene was <0.2% of that of the wild-type gene (Figure 5B). The CAT mRNA levels were similar in the pCATSUP-transfected cell lines, as determined by Northern analysis (data not shown). These data suggest an absence of functional edited $tRNA^{Trp}$(UCA) in the cytosol *in vivo* that could act as a suppressor of the UGA codon, and are consistent with the above primer extension/cell fractionation evidence for an intra-mitochondrial localization of the edited $tRNA^{Trp}$(UCA).

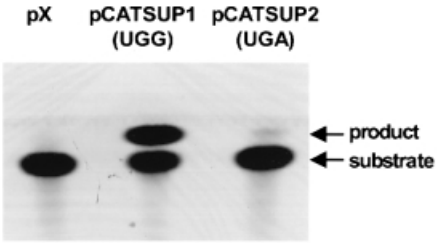
The C to U editing of $tRNA^{Trp}$ occurs after 3′-CCA addition

The $tRNA^{Trp}$ was amplified from kRNA by a 3′ tag RT–PCR method that preserved the 3′ end, and 30 independent clones were sequenced. As shown in Figure 5C, all 30 clones had 3′-CCA sequences, and 14 of these showed C to U editing at nucleotide 34, suggesting that editing occurs after 3′-terminal CCA addition.

Evidence for the absence of lysidine in the anticodon arm

Lysidine is a hypermodified cytosine that can base pair with adenosine and that would also allow decoding of the mitochondrial UGA codons (Maréchal-Drouard *et al.*, 1990). However, two-dimensional thin-layer chromatographic analysis of the P1 nuclease-digested 13mer T1 oligonucleotide that contains the anticodon showed no evidence for a lysidine spot (Figure 6). In addition, no lysidine residue was detected by mass spectrometry analysis of the purified 13mer oligonucleotide (P.Crain and J.McCloskey, personal communication).

A



B

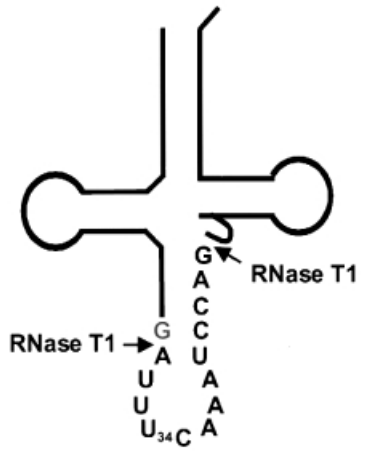
Plasmid	Specific activity (U/mg)	%
pX	0.06 + 0.02	–
pCATSUP1 (UGG)	29.58 + 0.71	100.0
pCATSUP2 (UGA)	0.11 + 0.02	0.2

C

	Number of Clones	
	+CCA	-CCA
Edited	14	0
Pre-edited	16	0

Fig. 5. Lack of suppression of a UGA codon in a nuclear transgene. (**A**) Thin-layer chromatography showing a CAT assay performed using extract from *L.tarentolae* expressing a wild-type CAT gene (pCATSUP1) or a mutated CAT gene containing a UGA codon in place of the UGG tryptophan codon (pCATSUP2). Cells transfected with the pX expression vector (LeBowitz *et al*., 1990) lacking the reporter gene were included to determine the experimental background. The pCATSUP1 reaction was incubated for 15 min, whereas the pX and the pCATSUP2 reactions were incubated for 2 h in order to visualize the product in the latter case. (**B**) Specific activity of CAT in cells transfected with the different plasmids. Mean values were calculated from three independent tests: 1 CAT unit is defined as the amount of enzyme that catalyzes the conversion of 1 nmol of substrate into product per min. The relative percentage activity was calculated after subtraction for background. (**C**) Summary of cDNA clones of RT–PCR-amplified $tRNA^{Trp}$. Thirty random clones were sequenced. The numbers of edited (with U34) or unedited (with C34) clones containing a 3′-terminal CCA sequence are indicated.

A



B

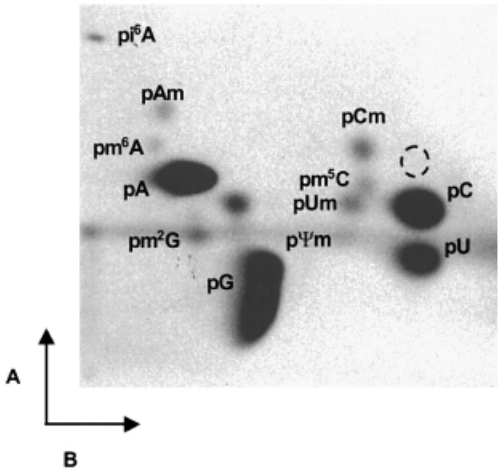


Fig. 6. Thin-layer chromatographic analysis of the anticodon arm 13mer oligonucleotide derived from affinity-purified mitochondrial $tRNA^{Trp}$. (**A**) Schematic diagram of the $tRNA^{Trp}$, showing the 13mer oligonucleotide released by RNase T1 digestion. The arrows indicate the RNase T1 cleavage sites on the 3′ side of guanosines. (**B**) Post-labeling analysis of the 13mer oligonucleotide showing the absence of lysidine, and the presence of other modified nucleotides. Arrows labeled A and B represent the two dimensions used during TLC, as described in Materials and methods. Abbreviations: pi^{6}A, N^6-isopentenyladenosine; pAm, 2′-*O*-methyladenosine; pm^{6}A, N^6-methyladenosine; pm^{2}G, N^2-methylguanosine; pCm, 2′-*O*-methylcytidine; pm^{5}C, 5-methylcytidine; pUm, 2′-*O*-methyluridine; pΨm, 2′-*O*-methylpseudouridine; pA, pC, pG and pU, unmodified 5′ ribonucleotides. The hatched circle represents the expected location of lysidine. All modification assignments are based on published maps (Sprinzl *et al*., 1986).

Discussion

In metazoa, the mitochondrial genome encodes a full set of tRNAs responsible for mitochondrial translation. However, in many protists (Suyama, 1986; Simpson *et al*., 1989; Hancock and Hajduk, 1990; Pritchard *et al*., 1990; Lye *et al*., 1993; Rusconi and Cech, 1996), fungi (Martin *et al*., 1979; Tarassov and Martin, 1996) and plants (Maréchal-Drouard *et al*., 1996), the mitochondrial genome lacks a complete set of tRNAs. The presumed loss of tRNA genes from the early protomitochondrion in these organisms has been compensated for by the development of a mechanism for the importation of nucleus-encoded tRNAs into the mitochondrion. There is *in vivo* and *in vitro* evidence for the mitochondrial importation of a single $tRNA^{Lys}$ in *S.cerevisiae* (Entelis *et al*., 1998), of multiple tRNAs in ciliate protists (Suyama, 1986; Pritchard *et al*., 1990; Gray *et al*., 1998), and all the tRNAs in the trypanosomatids (Simpson *et al*., 1989; Hancock and Hajduk, 1990).

In this study, we have presented evidence for C to U nucleotide modification editing at the first position of the anticodon of the nucleus-encoded mitochondrial imported $tRNA^{Trp}$(CCA). This editing event leads to the formation of a $tRNA^{Trp}$(UCA) that can decode the mitochondrial UGA codons as tryptophan. In other organisms, decoding of mitochondrial UGA codons by a $tRNA^{Trp}$(CCA) is proposed to occur by mutations outside the anticodon that permit aberrant base pairing between the first anticodon

position and the third codon position (C–A pairing) (Burger *et al*., 1995) or by a specific modification of cytosine (e.g. lysidine) that allows C–A pairing (Hirsh and Gold, 1971; Paquin *et al*., 1997). In the case of the *Leishmania* $tRNA^{Trp}$, the C to U editing of the anticodon is the only sequence change observed in an otherwise wild-type tRNA. This was determined by PCR amplifying the entire mature edited tRNA using a 3′ and 5′ oligonucleotide double-tagging method and sequencing the PCR product (S.Kapushoc, J.D.Alfonzo and L.Simpson, unpublished results). The presence of an edited mitochondrial $tRNA^{Trp}$(UCA) that lacks any other sequence change that could alter its canonical structure leads to the conclusion that tRNA editing is essential for the proper functioning of the $tRNA^{Trp}$ in mitochondrial translation.

The poisoned primer extension results in Figure 4 and the *in vivo* functional evidence for the absence of a UGA suppressor tRNA in Figure 5A and B both indicate that the editing reaction is localized in the mitochondrion. The evidence in Figure 5C that editing occurs after 3′-CCA addition may not be directly relevant to this problem, since we have recently found that 3′ end processing of this tRNA occurs prior to export from the nucleus (S.Kapushoc, J.D.Alfonzo and L.Simpson, unpublished results).

The evolutionary origin of the UGA tryptophan codon in different mitochondrial genetic systems (Barrell *et al*., 1980) and *Mycoplasma* spp. (Andachi *et al*., 1989) is proposed to involve ‘codon capture’ (Osawa *et al*., 1992; Osawa and Jukes, 1995), which has apparently occurred independently in several lineages (Inagaki *et al*., 1998). One of the proposed steps is a gene duplication event of a wild-type tRNA gene [e.g. $tRNA^{Trp}$(CCA)] followed by a point mutation in the anticodon that leads to the appearance of a new genomically encoded tRNA that can decode the UGA codon. Experimental evidence for a similar type of ‘tRNA gene recruitment’ in *Escherichia coli* has been presented (Saks *et al*., 1998) in which a mutation in the anticodon of a heterologous tRNA could rescue a strain in which an essential tRNA was deleted. The C to U editing of the *Leishmania* $tRNA^{Trp}$ and that described previously in a marsupial tRNA (Börner *et al*., 1996) offer an alternative scenario for the creation of a functional tRNA in the course of codon reassignment at the RNA level without a requirement for gene duplication. It is likely that the loss of tRNA genes from the mitochondrion and the importation of tRNAs from the cytosol occurred prior to the proposed ‘capture’ of UGA codons in the maxicircle genome. Once these codons appeared, there would be a requirement for a tRNA that could decode UGA as tryptophan, which was solved by C to U editing of the anticodon. Since C to U editing is observed today in many phylogenetically diverse organisms, both in the nucleus and in the mitochondria and plastids, it is likely that this was a pre-existing nucleotide modification reaction that was adopted to create a UCA anticodon in the imported $tRNA^{Trp}$. Consistent with this interpretation is a very recent report of C to U editing of the cytosolic 7SL RNA in the trypanosomatid *Leptomonas collosoma* (Ben Shlomo *et al*., 1999).

It is of some interest that 8% of the UGA tryptophan codons in the maxicircle genome are created by guide RNA (gRNA)-mediated U insertion editing. Therefore, a specific gRNA is required to insert a U to form a UGA codon that is then recognized by an imported tRNA, the anticodon of which is edited by a specific C to U modification. This raises the evolutionary question of which came first, gRNA-mediated U insertion editing or C to U editing?

Finally, it is unlikely that C to U editing in the mitochondrion of kinetoplastid protozoa is limited to a single imported tRNA, and the uncovering of the full extent of this phenomenon should prove an interesting challenge. The possible occurrence of C to U editing of maxicircle cryptogene mRNA transcripts and gRNAs remains to be investigated. It is clear that the discovery of a C to U editing system within kinetoplastid mitochondria opens up a number of interesting genetic and evolutionary questions.

Materials and methods

RT–PCR

Reverse transcription reactions were carried out using primer S2819 (5′-TGAGAGCTGCAGGGATTGTTCC-3′). This primer is complementary to the 3′ end of the $tRNA^{Trp}$ (nucleotides 72–57). The reverse transcription reaction included 5 µg of either total cellular RNA or total kRNA. The reverse transcription reaction was carried out as described in the SuperScript™ II reverse transcriptase first strand synthesis protocol (Gibco-BRL). Following reverse transcription, 1 µl of the 20 µl reverse transcription reaction was used as template in a PCR with primer S2819 (above) and primer S2820 (5′-GGGGGCTTAGCTCAGTGGTAGAGC-3′), corresponding to the 5′ end of the $tRNA^{Trp}$(CCA) sequence. PCR conditions were: 30 cycles of 95°C for 30 s, followed by 60°C and 72°C, each for 30 s. Each reaction contained 200 µM dNTPs, 1 U of *Taq* polymerase (Gibco-BRL), 1.8 mM $MgCl_2$, 10 µl of *Taq* polymerase buffer and 140 µM of each primer in a total reaction volume of 100 µl.

RT–PCR amplification of $tRNA^{Trp}$ for 3′ end analysis

A 20 µg sample of total mitochondrial tRNA was polyadenylated according to established methods (Sampson and Saks, 1996). The polyadenylated tRNA was used as a template for reverse transcription using a poly(A) tail-specific primer (RT oligonucleotide) 5′-TTGAATTCGCATTGAGCACCTGCTTTTTTTTTTTTTTTTT-3′. The cDNA (1 µl out of the 20 µl reverse transcription reaction) was used as template in a PCR as described above, with a primer specific for the anchoring sequence provided by the RT oligonucleotide (5′-TTGAATTCGCATTGAGCACCTGC-3′), and S2820 as the 5′ tRNA-specific primer. The product was cloned into PCR 2.1-TOPO (Invitrogen) and 30 random clones were sequenced.

DNA sequencing

Products from the RT–PCR above were cloned into PCR 2.1-TOPO (Invitrogen). Five clones from each reaction (RT+, RT– control and total DNA control, see Figure 1) were sequenced with Sequenase 2.1 following the manufacturer’s instructions (USB).

Poisoned primer extension analysis

Primer extension reactions were performed using SuperScript™ II reverse transcriptase (Gibco-BRL) as described (Estevez *et al*., 1999). The reactions included either 5 µg of total cellular RNA or 1 µg of total kRNA, and 1.25 mM ddGTP. The primer, 5′-TGAGAGCTGCAGGGATTGAACCTACGACCCCTGGATTTG-3′, complementary to nucleotides 72–34 in the tRNA sequence (or 73–35 in the canonical tRNA structure) (Figure 1B), was used in the primer extension reactions.

Cell culture and isolation of mitochondria and mitochondrial RNA

Leishmania tarentolae (UC strain) cells were grown in a New Brunswick Bioflow IV fermenter at 27°C in brain–heart infusion (BHI) medium (Difco) with 10 µg/ml hemin. Mitochondria were isolated as described (Braly *et al*., 1974) and total kRNA was isolated as described (Chomczynski and Sacchi, 1987). To obtain cytosolic RNA, which includes both cytosolic and nuclear RNA, the mitochondrial fraction

was removed from the preparation by ultracentrifugation at 100 000 *g* for 1 h prior to RNA isolation.

Construction of reporter plasmids and CAT assay

The CAT gene cassette was excised from the plasmid pT7CAT1 (Estevez *et al*., 1999) and ligated into the pX expression vector (LeBowitz *et al*., 1990), yielding pCATSUP1. pCATSUP2 was obtained by mutating the UGG tryptophan codon at position 16 of the CAT polypeptide to UGA. The *L.tarentolae* cells were electroporated with 5 μg of each plasmid and selected on BHI agar plates containing 200 μg/ml G418 (Gibco-BRL). CAT assays were performed using 1×10^7 cells with the FAST CAT (deoxy) chloramphenicol acetyltransferase assay kit (Molecular Probes), as recommended by the manufacturer. The amount of substrate converted into product during the CAT reactions was quantified using a Storm 860 blue fluorescence scanner and ImageQuant software (Molecular Dynamics).

Affinity purification of the mitochondrial $tRNA^{Trp}$

Total mitochondrial RNA was purified as previously described (Simpson and Simpson, 1978). Typically, 40 mg of total mitochondrial RNA were obtained from 90 l of exponentially growing cells. The total RNA was annealed to a 45 nucleotide biotinylated oligonucleotide (5′-biotin-TGGTGAGAGCTGCAGGGATTGAACCTACGACCCCTG) bound to streptavidin–agarose beads (Sigma). This oligonucleotide is antisense to the 3′ end of $tRNA^{Trp}$. Annealing was carried out by heating the RNA–biotin-oligomer–beads mixture at 70°C for 10 min followed by incubation at room temperature for 2 h in 6× SSC. The beads were then washed at room temperature with 6× SSC, followed by three washes with 3× SSC and exhaustive washes with 1× SSC. The efficiency of these washes was monitored by OD_{260} absorbance. The 1× SSC wash was continued until no OD_{260} absorbance was detected when using a similar buffer as blank. The affinity bound $tRNA^{Trp}$ was then eluted in 0.1× SSC, 0.1% SDS buffer at 70°C. The eluted samples were gel purified through consecutive 10 and 15% acrylamide–7 M urea gels. The tRNA band was excised and eluted at 4°C overnight in 0.3 M sodium acetate pH 5.2. The eluted RNA was precipitated by the addition of 3 vols of 100% ethanol and 20 μg of glycogen as a carrier.

Thin-layer chromatography analysis

A 13mer oligonucleotide was isolated by purification on a 7 M urea–15% acrylamide gel following RNase T1 digestion of the affinity-purified mitochondrial $tRNA^{Trp}$. RNase T1 digestion was carried out as described by the manufacturer (Ambion). This oligonucleotide contains the anticodon together with some flanking sequences (Figure 6A). The gel-purified oligonucleotide was dephosphorylated with calf intestinal phosphatase (Gibco-BRL) and digested with RNase T2 for 5 h at 37°C in 10 mM ammonium acetate buffer (pH 4.5). The resulting 3′ NMPs were labeled with T4 polynucleotide kinase for 45 min at 37°C in the appropriate buffer (Gibco-BRL). To remove unincorporated ATP, the mixture was treated with 5 U of apyrase (Sigma), and cold ATP was added to a final concentration of 1 mM. The mixture was incubated further at 37°C for 2 h. This treatment yields a mixture of 5′-labeled pNps. Following the apyrase treatment, the samples were treated with nuclease P1 (5 U/10 μl reaction) in 75 mM ammonium acetate (pH 5.3) buffer to remove the 3′ phosphates. The mixture was then extracted with chloroform, ethyl ether and dried in a SpeedVac (Savant). The pellet was resuspended in water, and ~20 000 c.p.m. were loaded onto a cellulose thin-layer chromatography plate (Merck) and analyzed by two-dimensional TLC. The nucleotides were separated using isobutyric acid, 25% ammonium hydroxide, water (50:1.1:28.9, by vol.) as the solvent system for the first dimension (solvent A in Figure 6B). The solvent system for the second dimension is 0.1 M sodium phosphate pH 6.8, ammonium sulfate, *n*-propanol (100:60:2, v/w/v). In addition, a second two-dimensional TLC system was used to confirm the nucleotide assignments. The same solvent system was used for the first dimension and a solvent system consisting of HCl, isopropanol and water (15:70:15, by vol.) was used for the second dimension. After chromatography, the plates were dried at room temperature and subjected to PhosphorImager analysis (Molecular Dynamics). Nucleotide assignments were made using published maps (Sprinzl *et al*., 1986).

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