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C to U Editing Stimulates A to I Editing in the Anticodon Loop of a Cytoplasmic Threonyl tRNA in *Trypanosoma brucei*[*][S]

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Editing of tRNAs is widespread in nature and either changes the decoding properties or restores the folding of a tRNA. Unlike the phylogenetically disperse adenosine (A) to inosine (I) editing, cytosine (C) to uridine (U) editing has only been previously described in organellar tRNAs. We have shown that cytoplasmic tRNA^{Thr}(AGU) undergoes two distinct editing events in the anticodon loop: C to U and A to I. *In vivo*, every inosine-containing tRNA^{Thr} is also C to U edited at position 32. *In vitro*, C to U editing stimulates conversion of A to I at the wobble base. Although the *in vivo* and *in vitro* requirements differ, in both cases, the C to U change plays a key role in A to I editing. Due to an unusual abundance of A34-containing tRNAs, our results also suggest that the unedited and edited tRNAs are functional, each dedicated to decoding a specific threonine codon. C to U editing of cytoplasmic tRNA expands the editing repertoire in eukaryotic cells, and when coupled to A to I changes, leads to an interrelation between editing sites.

The degeneracy of the genetic code is implied in the need for 61 sense codons to specify 20 different amino acids and, with the exception of methionine and tryptophan, each amino acid is encoded by more than one codon (1). This discrepancy between codon and amino acid numbers was first explained by Crick's wobble hypothesis, which invoked flexibility between the first anticodon and third codon positions during decoding (2). Since the inception of the wobble rules, over 100 posttranscriptional modifications have been described with the largest number affecting the anticodon of tRNA (3, 4). As anticodon modifications accrue, new findings lead to a constant reinterpretation of the wobble rules to include novel effects on tRNA function. Although some anticodon modifications play key roles in translational fidelity and efficiency (1, 5), anticodon-sequence alterations that permit decoding of multiple codons are part of a growing number of posttranscriptional changes collectively known as tRNA editing. Thus decoding changes imparted by tRNA editing provide a mechanism to effectively accommodate genetic code degeneracy. To date, well characterized anticodon editing events include editing of C34 to lysidine of methionyl tRNAs in bacteria, which permits decoding of AUA codons as isoleucine (6, 7), cytidine (C) to uridine (U) editing in eukarya, which reassigns tRNA^{Gly} and tRNA^{Trp} to new codons in mitochondria (8, 9), and adenosine (A) to inosine (I) editing, which expands tRNA decoding capacity and is found in organisms from each of the three domains of life (4, 10).

Although inosine was first discovered over 40 years ago in tRNA (11), its involvement in codon alterations in eukaryotes was first demonstrated by the discovery of A to I editing in mRNAs (12, 13). Inosine in mRNA expands the number of proteins that can be encoded from a single gene and is a significant source of genetic diversity (14). In tRNA, adenosines at the first position of the anticodon (A34, wobble position) are almost universally changed to inosine by hydrolytic deamination of the 6-amino group of the base (4, 15). This editing reaction is so efficient that under steady-state conditions, A34-containing tRNAs are difficult to detect, tRNA^{Thr} from *Mycoplasma*, thus far, being the only naturally occurring exception (16).

C to U editing of tRNA is less prevalent and, until now, restricted to eukaryotic organelles. In marsupial mitochondria, a single C to U editing event at the second position of the anticodon (C35) changes a tRNA such that it recognizes aspartate in place of glycine codons (9, 17–19). Some evidence supports the requirement for methylation and pseudouridylation reactions prior to editing (19). C to U editing in this system is also required for creating the proper substrate for further modification of the first anticodon position (G34) (19). This C to U editing also generates structural features important for aminoacyl-tRNA synthetase recognition (9). The only other example of C to U editing of tRNA occurs in the mitochondria of trypanosomatids (8), where the nucleus-encoded tryptophanyl tRNA (tRNA^{Trp}) is transcribed with a CCA anticodon. A subpopulation of this tRNA is imported into the mitochondrion, where RNA editing of C_{34} creates the U_{34}CA anticodon required to translate the UGA tryptophan codons found in mitochondrial mRNAs. Following mitochondrial import, tRNA^{Trp} undergoes an unprecedented number of posttranscriptional modifications, which, as in the marsupial system, may play a role in editing specificity (8, 20).

In the current study, we have shown that the cytoplasmic tRNA^{Thr}(AGU) of *Trypanosoma brucei* undergoes two distinct editing events in the anticodon loop, where A34 is changed to inosine and C32 is changed to uridine. We demonstrated that C to U editing at position 32 affects the efficiency of A to I editing of the anticodon. These findings represented the first example of C to U editing of tRNAs outside organelles and demonstrated an interrelation between two different editing sites in a single anticodon loop. Unlike most organisms, we also reported an abundance of unedited tRNAs, which are substrates for aminoacylation *in vivo*. Together, our findings have raised new and important questions about the prevalence of tRNA editing in eukaryotes and demonstrated a functional role for double editing of tRNAs in trypanosomatids.

MATERIALS AND METHODS

Cell Culture and Preparation of Cell-free Extracts—T. brucei cells were grown in SDM-79 medium supplemented with 10% fetal bovine serum (Fisher) and 10 g/ml hemin (Calbiochem). Exponentially grow-

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ing cultures (2×10^6 cells/ml) were harvested by centrifugation at 4,000 × *g* and washed with phosphate-buffered saline. The resulting pellets were suspended in buffer containing 50 mM Hepes, pH 8.0, 50 mM KCl, 2.5 mM EDTA, 1 mM DTT.[3] The suspension was sonicated with a Sonifier 450 sonicator (Branson) using a microprobe at 50% output for a total of five intervals with 1-min rest between sonication. The resulting lysate was initially spun at 10,000 rpm in a Beckman Coulter Avanti J-25 centrifuge JA25.50 rotor for 15 min at 4 °C followed by a 30-min centrifugation at 100,000 × *g* in a Beckman Coulter Optima L-90K ultracentrifuge Type 60TI rotor at 4 °C. To the clarified lysate, glycerol was added to a final concentration of 20% and stored frozen in 4 mg/ml aliquots at −80 °C.

cDNA Synthesis and Amplification by PCR—RNA was isolated from cells (total RNA) and/or nuclear fractions by the guanidinium thiocyanate/phenol/chloroform extraction method (21) and as described previously by us (22). RNA was further treated with RQ1 (RNA qualified) RNase-free DNase I (Promega). Two picomoles of reverse oligonucleotide primer (57R: 5′-AGGCCACTGGGGGGATCGAACCC-3′) complementary to the 3′-end of $tRNA^{Thr}$(AGU) was added to 5 μg of total or nuclear RNA with 10 μmol of all four deoxynucleotide triphosphates and heated at 65 °C for 5 min and then quick-cooled at 4 °C for 1 min followed by the addition of 1 μl of SuperScript™ II reverse transcriptase-(RT) in 1× first strand buffer and incubation at 50 °C, as described (Invitrogen). Following the RT reaction, the cDNA was amplified from 2 μl of the 20-μl RT reaction as a template in a 100-μl (PCR) with 40 pmol of forward (56F: 5′-GGCCGCTTAGCTCAATGGCAGAG-3′) and 40 pmol of reverse (57R) oligonucleotide primers. PCR reactions were performed using *Taq* DNA polymerase and incubated in a thermal cycler using a program consisting of a 94 °C denaturation step, a 50 °C annealing step for 40 s, and an elongation step of 72 °C repeated for a total of 20 cycles, following manufacturer's instructions (PerkinElmer Life Sciences). Controls included a mock reaction in which the RT was left out of the reaction and used as a negative control to test for DNA contamination in the RNA samples and a reaction in which total genomic DNA was used as a template serving as a positive control for amplification. RT-PCR products were cloned into pCR2.1-TOPO (Invitrogen). Independent clones were isolated after transformation of DH5α *Escherichia coli* and sequenced using Sequenase™ Version 2.0 DNA polymerase (USB), per the manufacturer's instructions. The dideoxynucleotide terminated sequencing reactions were separated in a 6% acrylanmide/7 M urea denaturing gel, and the resulting sequences were used to ascertain the state of editing for each clone.

In Vitro Editing Assays—In vitro transcribed tRNAs with internally incorporated [α-^{32}P]ATP were heated in water at 70 °C for 3 min and allowed to cool to room temperature. After 1 min, reaction buffer was added to a final concentration of 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 2.5 mM $MgSO_4$, 0.1 mM EDTA (pH 8.0), 2 mM dithiothreitol), and the mixture was allowed to cool for an additional 5 min. The reaction was started by the addition of cell extract and incubated at 27 °C. For the time course experiments, a large reaction (446 μl) containing 40 pmol of RNA (200,000 cpm) in reaction buffer was assembled. As a negative control, an aliquot of 49 μl was transferred into a separate tube and incubated at 27 °C for 480 min. To the remaining 397-μl reaction mix, 8.1 μl of cell extract was added and incubated at 27 °C. Eight individual aliquots of 50 μl were removed after 1, 30, 50, 120, 180, 240, 360, and 480 min, respectively). Each sample was extracted using an equal volume of phenol (previously saturated with 10 mM Tris-HCl, pH 8). The RNA in the aqueous phase was recovered after precipitation with a 0.1 volume equivalent of 3 M sodium acetate (pH 5.2), 2.5 volumes of ethanol and incubated at −20 °C. After centrifugation, the resulting pellet was dissolved in 30 mM ammonium acetate and 10 mM zinc acetate containing 0.4 units nuclease P1 in a 20-μl reaction (MPBiomedicals). The digestion reaction was incubated at 37 °C for at least 12 h. The reaction was dried down in a SpeedVac DNA 110 concentrator system (Savant) for 10 min under high heat. The dried sample was resuspended in 3 μl of double distilled H_2O, where 1 μl (13.33 pmol) was spotted and dried individually onto a cellulose TLC sheet (EMD Chemicals). On the same sheet, 2.5 pmol of a cold mix containing adenosine 5′-monophosphate and inosine 5′-monophosphate was spotted in a separate lane and used as cold markers. The TLC was allowed to develop using liquid chromatography in Solvent C (0.1 M sodium phosphate (pH 6.8):ammonium sulfate:*n*-propyl alcohol (100:60:2, v/w/v). The TLC plate was allowed to dry and was then exposed to a PhosphorImager™ screen. The resulting images were visualized and quantified using an Amersham Biosciences Storm® imaging system with an ImageQuant® program (Amersham Biosciences). Cold markers were visualized by a hand-held ultraviolet lamp at 260 nm and used to assess the relative migration of the ^{32}P-labeled individual nucleoside 5′-monophosphates from the radiolabeled samples. Two-dimensional TLC was used to further confirm the relative positions of nucleoside 5′-monophosphates assignments. The first dimension of the TLC plate was developed in Solvent A (isobutyric acid:25% ammonium hydroxide:H_2O; 50:1.1:28.9, v/v/v). The TLC plate was removed and allowed to dry before separation in the second dimension by developing in Solvent B (isopropyl alcohol:concentrated HCl: water, 68:18:14, v/v/v) or solvent C (0.1 M sodium phosphate (pH 6.8): ammonium sulfate:*n*-propyl alcohol, 100:60:2, v/w/v). Nucleotide assignments were made using published maps (23).

In Vitro Aminoacylation and Oxidation Assays—To corroborate the editing state of aminoacylated species, total aminoacyl-tRNAs were extracted under acidic conditions (using phenol equilibrated with 0.3 M sodium acetate, pH 4.5, and 10 mM EDTA), ethanol-precipitated, and resuspended in 10 mM sodium acetate, pH 4.5, and 1 mM EDTA. The RNA was then split into two fractions. One fraction was deacylated by incubation at 37 °C for 1 h in a basic buffer (10 mM Tris, pH 9.0) followed by oxidation of the 3′-ribose by treatment with 40 mM $NaIPO_4$ in ice for 90 min. The second fraction was directly oxidated by $NaIPO_4$ followed by deacylation as above. Both fractions were individually polyadenylated by incubation of the RNA at 37 °C for 45 min in buffer containing 20 mM Tris, pH 7.0, 50 mM KCl, 0.7 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10% glycerol, 500 μM ATP and 1,700 units of yeast poly-A polymerase in 100 μl of reaction buffer. The reaction was then supplemented with 30 μl of 5× *E. coli* poly-A buffer (200 mM Tris, 7.0, 1 M NaCl, and 25 mM $MgCl_2$), 15 μl of 5 mM ATP, 1 μl of 0.1 M DTT, 3.5 μl of $MnCl_2$, and 3 units of *E. coli* poly-A polymerase and incubated further for 45 min at 37 °C. The reactions were phenol-extracted and ethanol-precipitated. Both reactions were then used in RT-PCR reactions. First, a 3′-primer specific for the poly-A tail was used to RT-PCR the poly-adenylated RNA followed by PCR with the RT primer and a 5′-specific primer specific for $tRNA^{Thr}$(AGU) in a 100-μl PCR reaction as above. One μl of this reaction was used as a template for a second PCR reaction in which both primers were specific for $tRNA^{Thr}$-(AGU). The resulting product was purified, cloned into pCR2.1-TOPO (Invitrogen), and transformed into *E. coli*, and individual clones were sequenced to establish editing levels.

For *in vitro* aminoacylation, all assays were performed at 37 °C as follows. A 35-μl pre-reaction mixture was first prepared containing 100 mM Hepes (pH 7.5), 25 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 5 mM DTT, 15 μM *in vitro* transcribed $tRNA^{Thr}$ variants (see "Results" for details),

[3] The abbreviations used are: DTT, dithiothreitol; RT, reverse transcriptase.

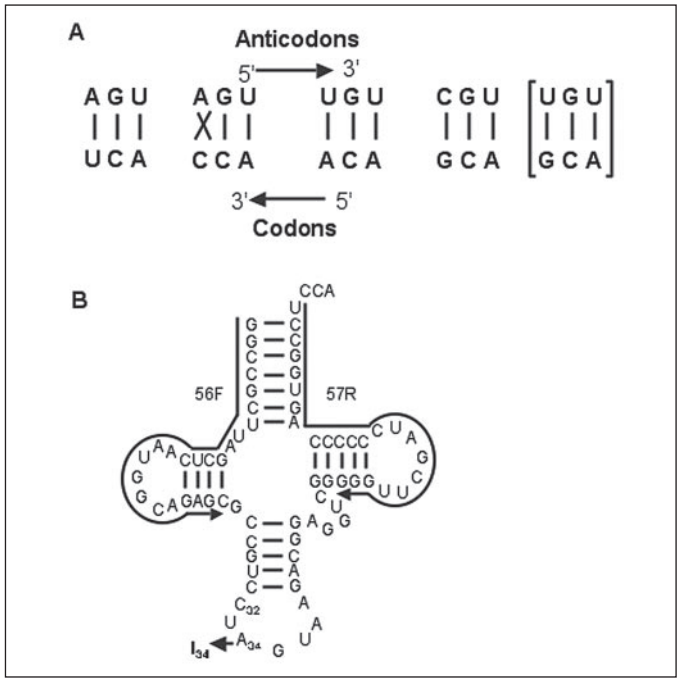
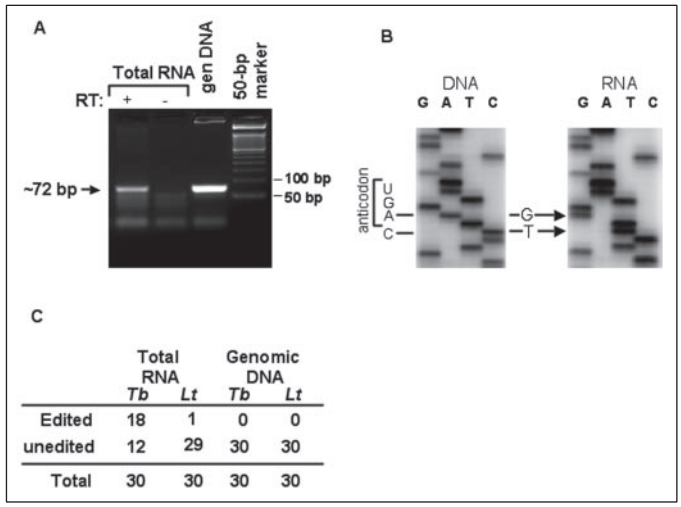


FIGURE 1. **A to I editing of tRNA^{Thr}(AGU) allows decoding of the C-ending threonine codon.** *A*, the four threonine codons used in trypanosomatid translation and their respective tRNAs. A possible decoding of the GCA codon by UGU wobbling is shown in *brackets*. The *arrows* indicate the sequence polarity. Isoaccepting tRNAs, which may decode the ACU, ACA, and ACG codons, are genomically encoded. No tRNA that may decode the remaining codon (ACC) is encoded in the genome and must be formed by editing. *B*, tRNA^{Thr}(AGU) is proposed to undergo A to I editing, where the wobble position inosine can then decode the remaining threonine codon by wobbling. *Arrows* indicate the position of the primers (56F and 57R) used in the RT and PCR reactions. The *short arrow* denotes the edited position.



C

	Total RNA		Genomic DNA	
	Tb	*Lt*	*Tb*	*Lt*
Edited	18	1	0	0
unedited	12	29	30	30
Total	30	30	30	30

FIGURE 2. **tRNA^{Thr}(AGU) undergoes two different editing events in the same anticodon loop.** *A*, RT-PCR analysis of tRNA^{Thr}(AGU) and PCR analysis of genomic DNA. *RT+* refers to reverse transcription reactions using a tRNA^{Thr}(AGU)-specific primer and total *T. brucei* RNA. *RT−* is a negative control in which the reverse transcriptase was left out of the reaction. *gen DNA* refers to a PCR reaction (positive control) using the same oligonucleotides above, and *marker* refers to a 50-bp DNA size marker. The 72-bp band is of the size expected for a product from PCR or RT-PCR reactions using the primers specific for tRNA^{Thr}(AGU) as per Fig. 1. *B*, representative sequencing gels from independent isolates generated by cloning the products from *A* into a plasmid. The *arrows* denote the two-nucleotide changes observed in the cDNA sequences derived from total tRNA but absent from amplified genomic DNA. *C*, a summary table of the results of sequencing 30 independent clones derived from cDNA copies (total RNA) or genomic DNA copies. *Edited* refers to the presence of both editing events. Conditions for the different reactions above are as described under "Materials and Methods." *Tb*, *T. brucei*. *LT*, *Leishmania tarentolae*.

and 28 μM L-[3-^{3}H]threonine. The reaction was started by the addition of 10 μl (1 μg/μl total protein) of *T. brucei* extract. Eight-μl aliquots were removed periodically and spotted onto 3MM filter disks presoaked in 5% trichloroacetic acid (w/v), washed three times in 5% trichloroacetic acid (w/v), rinsed in ethanol, and dried, and the remaining radioactivity was quantified by scintillation counting.

RESULTS

In the search for examples of interdependence between different editing sites, we have focused on the formation of inosine at the wobble base (position 34) of tRNAs in *T. brucei*. In these organisms, eight different tRNA species contain an encoded A at the first position of the anticodon (A34). These tRNAs are proposed to undergo A to I editing to allow the decoding of the C-ending codons for the amino acids Ile, Ala, Leu, Pro, Val, Ser, Arg, and Thr (see Supplementary Fig. 1). We decided to determine the A to I editing levels of threonyl tRNA. The genome of *T. brucei* encodes three different tRNA^{Thr} genes with anticodons UGU, CGU, and AGU responsible for decoding ACA, ACG, and ACU codons, respectively. As in most organisms, trypanosomatids use a fourth threonine codon (ACC), which presumably cannot be decoded by tRNA^{Thr}(AGU), because A34 cannot efficiently wobble with C at the third codon position (Fig. 1*A*). To permit wobbling, this tRNA must undergo an A to I editing at position 34, where inosine (a guanosine analog) can then pair with the third position C (Fig. 1). To determine the levels of A to I editing *in vivo*, we designed oligonucleotide primers specific for tRNA^{Thr}(AGU) (Fig. 1*B*), where a 3′-specific oligomer was used to reverse-transcribe tRNA^{Thr} from total *T. brucei* RNA. The resulting cDNA was then used as a template for PCR amplification with the same 3′-primer and a 5′-specific oligomer. A specific amplification product was obtained with this set of primers when the reaction was performed in the presence of reverse transcriptase (Fig. 2*A*) but was absent in a mock control in which the enzyme was omitted from the reaction. A product of identical size was obtained when both primers were used to amplify tRNA^{Thr} from total genomic DNA used as a positive control for amplification (Fig. 2*A*). Both the cDNA-derived and the genomic DNA-derived products were then cloned into a plasmid vector and transformed into *E. coli*, and 30 independent clones were sequenced to assess the levels of A to I editing. As expected, we found that A34 is posttranscriptionally changed to I34 (G34 in the DNA sequence), where 18 out of 30 clones (60%) contained a G at position 34 (corresponding to inosine in the RNA sequence) (Fig. 2, *B* and *C*). These sequences also showed a second editing event at position 32 of the same tRNA. Position 32 is a genomically encoded cytidine, which as shown here is posttranscriptionally changed to uridine (Fig. 2*B*). In fact, under the conditions described, all of the inosine-containing tRNAs also have the C to U change (Fig. 2*C*). These results demonstrate the first example of two different editing events in a single tRNA in any organism. A similar type of editing, but at much lower levels, was also observed with *L. tarentolae*, a slose relative of *T. brucei*.

The findings above raised questions as to a possible connection between the two processes. First, we tested for A to I editing *in vitro*. A ^{32}P-labeled tRNA^{Thr}(AGU) was generated by *in vitro* transcription whereby every adenosine is radioactively labeled. This substrate was then incubated for various times with total cell-free extracts from *T. brucei*. Following this incubation, the labeled tRNA was gel-purified followed by digestion with nuclease P1. The nucleotide mixture generated by the nuclease treatment was then separated by TLC as described previously (8). Unlabeled adenosine and inosine were used as cold markers during the TLC separation. These markers, when visualized by UV shadowing, serve to corroborate the position of labeled adenosine and inosine generated during the assay. We found that >50% of aden-

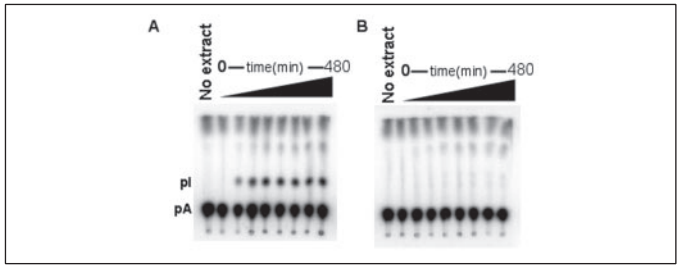


FIGURE 3. **tRNA^{Thr}(AGU) is efficiently edited *in vitro*.** tRNA^{Thr}(AGU) was labeled by *in vitro* transcription in the presence of [α-^{32}P]ATP. The labeled tRNA was incubated with total *T. brucei* extracts for increasing lengths of time followed by nuclease P1 digestion and separation by TLC. *A*, TLC analysis of the reaction in the presence or absence of extract, where *pA* and *pI* denote the positions of unlabeled 5′-AMP and 5′-IMP used as markers and visualized by UV shadowing (not shown). *B*, a reaction where a similar tRNA as in *A* but containing a G34 was used as a control for specificity. This reaction also served as a background control. The relative fraction of pA converted to pI was calculated by dividing the amount of radioactivity in the pI spot by the sum of the radioactivity in the pA+pI spots (pI/pI+pA). The specific percent conversion at a single site (*i.e.* A34) was then calculated by normalizing the amount of radioactivity at A34 to the total number of label adenosines ($n = 14$), where conversion at one site will yield a maximum theoretical value of 7.7% (or $^1/_{13}$ possible adenosines). The specific yield of pI was then calculated by dividing the percent total by the relative percentage at one site or $\%pI_{34}/\%pA_{34} \times 100$, where the theoretical maximum of 7.1% equals 100% conversion A to I conversion at position 34.

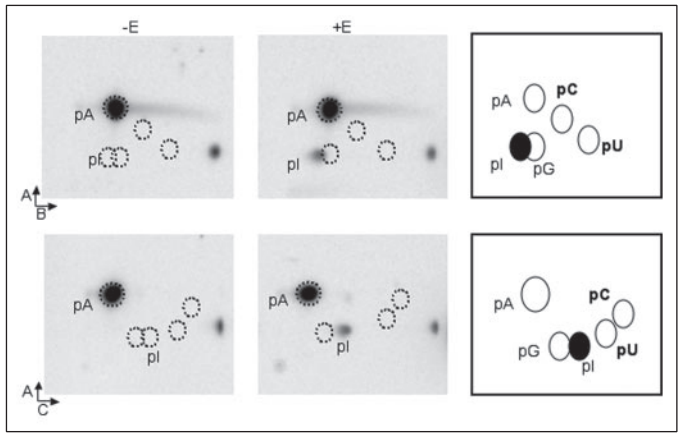


FIGURE 4. **Two-dimensional-TLC analysis of an *in vitro* A to I editing reaction.** Radioactive tRNA^{Thr}(AGU), where every adenosine is labeled, was used as a substrate in an *in vitro* editing assay by incubation with total *T. brucei* extract as described under "Materials and Methods." Following incubation, the tRNA was digested to nucleotides, and the products were separated on two-dimensional thin-layer chromatography. $-E$ and $+E$ refer to reactions performed in the absence or presence of extract, respectively. *pA*, *pG*, *pC*, *pU*, and *pI* refer to the migration of nucleotides as corroborated by comparison with published maps and/or by separation of individual nucleotides used as cold markers and visualized by UV shadowing (not shown) also depicted by *dashed circles*. *Arrows* denote the direction of migration during TLC. *A–C* refer to the different solvents used during chromatography as described under "Materials and Methods."

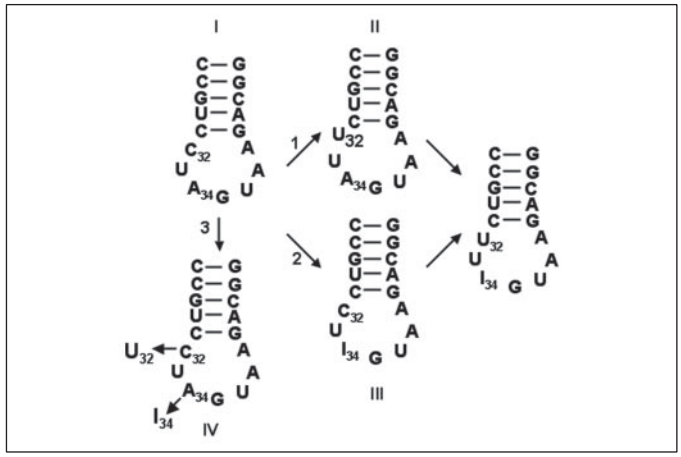


FIGURE 5. **An interdependence model for the double editing of tRNA^{Thr}.** In the event of interdependence, tRNA maturation may take one of two paths, indicated by ***1*** and ***2***. ***1***) either U32 is first converted into C32, and that affects A34 to I 34 conversion, or ***2***) A34 is converted to I34 first followed by the C32 to U32 conversion. If no interdependence occurs between the sites then ***3*** will prevail.

osine 34 was efficiently converted to inosine by the *T. brucei* extract under the assay conditions described (Figs. 3*A* and 6*C*, and data not shown). No detectable inosine was observed in a control reaction using a substrate in which A34 was changed to G34, indicating that the observed A to I conversion is specific for position 34 (Fig. 3*B*). A two-dimensional-TLC was also performed to confirm the identity of the reaction products (Fig. 4).

We recently proposed an interdependence model to explain the connection between editing and modification of tRNA^{Trp} in trypanosomatid mitochondria (24). We have now expanded this model to include cytoplasmic tRNAs in these organisms. We propose that double editing of tRNA^{Thr} occurs in a sequential manner, where editing at one position affects subsequent editing at a second position (Fig. 5). To test a possible connection between the two sites, we created *in vitro* transcribed tRNA substrates representing the unedited tRNA and a possible intermediate in the editing reaction (Fig. 5, *I* and *II*) so that every adenosine in the various tRNAs is radioactively labeled. Upon incubation of the different substrates with cell-free extracts, we found that the C32-containing substrate could support editing; however, a similar substrate in which C32 was replaced by U32 (Fig. 5, *II*) supported editing with reproducibly higher efficiency and significantly higher initial rate (compare Fig. 3*A* and Fig. 6, *A* and *C*). We also found that tRNA substrates, in which C32 was replaced by A or G, showed no stimulation (data not shown). Taken together, the *in vivo* observation that every inosine-containing tRNA^{Thr} is also edited at position 32 and the observed *in vitro* stimulation of inosine formation led us to conclude that editing at one site affects editing at a second site and that indeed, the two editing events are interrelated.

In our model, editing at position 32 occurs first, and it promotes efficient A to I editing at position 34 of the anticodon. If C to U editing at position 32 occurs first, this may impart subtle changes in the loop structure, providing the proper substrate for further editing at position 34 (Fig. 5, *I*). In this scheme, C to U editing may also affect tRNA aminoacylation in addition to modulating the ability of the tRNA to undergo further A to I editing. Alternatively, C to U editing may affect translational efficiency by affecting A to I formation, thus regulating wobbling. Recent evidence supports a role for I34 as a key determinant for synthetase recognition of tRNA^{Ile} in yeast (25). To test the possibility that A to I editing affects charging of tRNA^{Thr}, substrates were generated corresponding to the two partially edited intermediates (Fig. 5, *I* and *II*) and incubated with partially purified synthetase fractions from *T. brucei* in the presence of ^{3}H-labeled threonine. We found no significant difference in aminoacylation efficiency when the *in vitro* transcripts were compared with native tRNA (see Supplementary Fig. 2). However, *in vitro*, the presence of C32, in the A34-containing tRNA (the unedited tRNA), supported a reproducible 2-fold difference in amino acylation when compared with a similar substrate with a U at position 32. Interestingly, similar experiments performed with either substrate but with a G at position 34 supported similar charging efficiencies as the unedited tRNA (Fig. 7). The observed *in vitro* aminoacylation efficiency rules out the possibility that the differences in editing levels *in vitro* between the various substrates could be due to problems in the global folding of the *in vitro* transcribed tRNA substrates when compared with native substrates.

To further assess the editing state of the aminoacylated tRNAs *in vivo*, we designed a coupled oxidation/polyadenylation assay. In this assay,

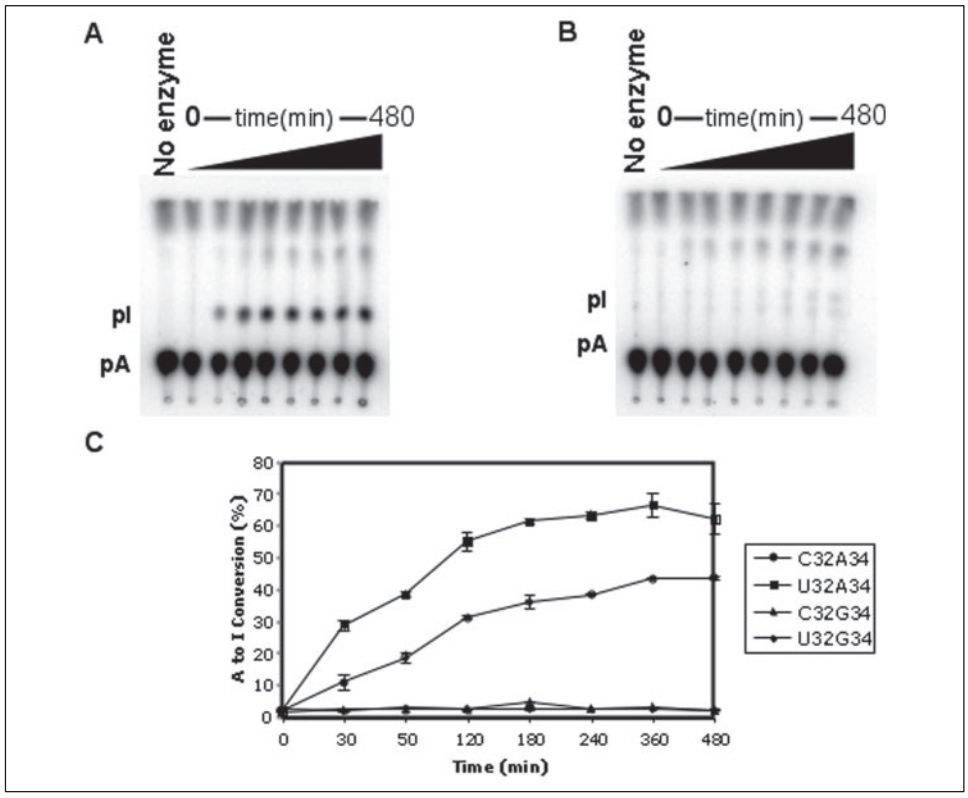


FIGURE 6. **The presence of U32 stimulates A to I conversion at the wobble base.** An [α-^{32}P]ATP-labeled pre-edited tRNA^{Thr}(AGU), where C32 was replaced by U32, was incubated with *T. brucei* extracts for various times. *A*, TLC analysis of an [α-^{32}P]ATP labeled U32-containing tRNA in the presence or absence of enzyme and increasing incubation times. *B*, TLC analysis of an [α-^{32}P]ATP labeled U32-containing tRNA in which A34 was replaced by G34 used as a control for specificity. *C*, a plot of the percentage of conversion of A to I at 34 *versus* time. The products of the reaction and calculations of percent conversion are as described in the legend for Fig. 3. The values shown are the result of five independent experiments, where values were averaged to obtain a measure of error between experiments and data points (error bars). *pI* and *pA*, refer to unlabeled inosine-5-monophosphate and adenosine-5-monophosphate used as markers for TLC.

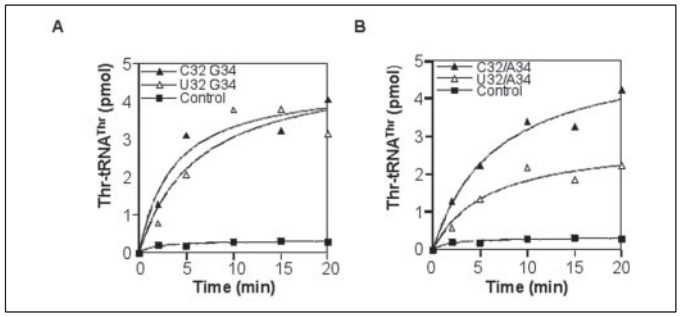


FIGURE 7. **tRNA^{Thr}(AGU) is efficiently aminoacylated *in vitro* regardless of its editing state.** Different versions of tRNA^{Thr}(AGU) were generated by *in vitro* transcription and then used in aminoacylation reactions in the presence of L-[3-^{3}H]threonine and partially purified synthetase fractions from *T. brucei*. *A*, a tRNA containing a G34 and either the pre-edited C32 or the edited U32 was used during the reaction. *B*, a similar experiment as in *A*, but the reactions were performed with tRNA substrates that are pre-edited at position 34 (A34) and either a pre-edited or an edited position 32 (C32 or U32). *Control* refers to a reaction where the *in vitro* transcript was left out of the reaction. This control serves as background during quantitation; *pmol* refers to the amounts of Thr-tRNA^{Thr} generated at various times expressed in picomoles.

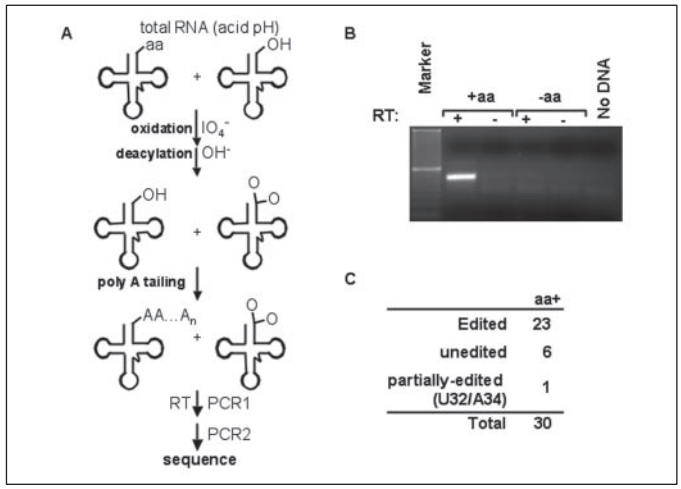


FIGURE 8. **The edited and pre-edited substrates are functional substrates for aminoacylation *in vivo*.** Total RNA was isolated from *T. brucei* under acidic conditions, oxidized, and polyadenylated, and the resulting template was used for RT-PCR to determine the editing state of the aminoacylated species. *A*, a scheme of the oxidation/tailing reaction. IO_4^- refers to a reaction where total acidic RNA was treated with sodium periodate. OH^- refers to the incubation of the acidic RNA at pH 9.0 for 30 min, leading to deacylation. *RT* refers to a cDNA synthesis reaction using the tailed tRNA or a negative control where the RNA was deacylated prior to oxidation. *PCR1* and *PCR2* are reactions in which the cDNA from the previous step was first used as a template for PCR using primers specific for the poly(A) tail and tRNA^{Thr} (*PCR1*) followed by a PCR reaction with tRNA^{Thr}-specific primers (*PCR2*). These reactions were performed in succession. *aa*, aminoacyl. *B*, the reaction products from *A* were separated on a 4% agarose gel and visualized by staining with ethidium bromide. *RT*+ and *RT*− refer to reactions performed in the presence or absence or reverse transcriptase as described. *Marker* refers to the 10-bp size marker used during electrophoresis. *C*, results of sequencing 30 independent clones, where *Edited* refers to double-edited tRNAs.

RNA fractions were isolated under acidic conditions and then subjected to a combination of *in vitro* oxidation, polyadenylation, and RT-PCR (see "Materials and Methods"). In these reactions, oxidation by sodium periodate led to formation of a dialdehyde at the 3′-end of uncharged tRNAs, whereas the 3′-end of aminoacylated tRNAs is protected from oxidation by the covalently attached amino acid. The oxidized tRNA is not a substrate for polyadenylation, whereas following deacylation, only the charged tRNA will have an intact 3′-end and will thus serve as a substrate for poly-A polymerase (Fig. 8*A*). Under these conditions, we observed an RT-PCR product when total RNA was oxidized as described, whereas no product was detected in a similar reaction, where the total RNA was deacylated prior to oxidation to de-protect every tRNA present in the mixture (Fig. 8*B*). The product from the reaction above was then purified, cloned, and sequenced. We found that, as in the *in vitro* situation, both the edited and the unedited tRNA were substrates for aminoacylation, where the majority of the charged species (23 out of 30 clones) corresponded to that of the double-edited tRNA (Fig. 8*C*).

DISCUSSION

We previously proposed an interdependence model for editing and modification in tRNA (24). This model suggests that editing and modifications at multiple sites act in concert to help achieve the degree of substrate specificity that different systems demand. To further expand this model, we have focused on the process of inosine formation in the tRNAs of trypanosomatids. Here we have described the first example of two different editing events in a single tRNA anticodon loop, whereby positions 32 and 34 of $tRNA^{Thr}$ undergo C to U and A to I editing, respectively. The finding that every inosine-containing tRNA also undergoes C to U editing at position 32 (5′ of the wobble position) raised important questions as to what role the two editing events play in the function of this tRNA. *In vivo*, every I34-containing $tRNA^{Thr}$(AGU) also has the C to U change at position 32. By establishing an A to I editing assay, we have demonstrated that C to U stimulates A to I editing *in vitro*, indicating interdependence between the two editing sites. The fact that the C32-containing *in vitro* transcribed tRNA (devoid of naturally occurring posttranscriptional modifications) can still be edited *in vitro* raises the possibility that in addition to C to U editing, other factors *in vivo* (*e.g.* posttranscriptional modifications) make the requirements for double editing stricter. The observed interdependence also suggested that these cells might be able to regulate A to I editing of $tRNA^{Thr}$ through changes in C to U editing activity.

A to I editing is conserved in many organisms and occurs by hydrolytic deamination of adenosine (26). In yeast, the enzyme involved has two subunits, resembling cytidine deaminases, that upon association specifically deaminates A34 to generate I34 and requires an intact tRNA structure for activity (26, 27). *E. coli* contains a homolog of the smaller, but not the larger, subunit of the yeast enzyme (28). The *E. coli* enzyme catalyzes the same A to I editing but, unlike the yeast enzyme, deaminates much smaller substrates, including molecules that are essentially short versions of the anticodon stem-loop (27–29). The *E. coli* enzyme, however, is very specific in that it is able to deaminate the cognate bacterial $tRNA^{Arg}$ but is unable to edit any of the eukaryotic A34-containing tRNAs (26, 28). Although the enzyme that performs A to I editing in trypanosomatids has not been identified, it is likely that this enzyme also utilizes a similar mechanism to that described for yeast and *E. coli*. The mechanism of C to U editing at position 32 is less clear. To date, a C to U tRNA editing enzyme has not been identified in any organism. However, C to U editing via a deamination mechanism plays a key role in the processing of the apoB mRNA in mammalian cells (30–32). Given this precedent, it is possible that the tRNA C to U editing enzyme also utilizes a deamination mechanism. Furthermore, in the case of ribose methylation, one enzyme, TRM7, is responsible for the methylation of both positions 32 and 34 in yeast tRNAs (33). A similar situation may have arisen in the double editing of $tRNA^{Thr}$ in trypanosomatids, where a single tRNA could be edited twice in the anticodon loop by a single deaminase with multisite and multinucleotide specificity.

Although the analysis of $tRNA^{Thr}$(AGU) in *T. brucei* confirmed the presence of the two editing events, it also revealed that under steady-state conditions, one could detect A34-containing tRNAs. This is unlike other organisms, where the A to I reaction occurs so efficiently *in vivo* that the levels of the A34 intermediate are difficult to detect. The fact that both the *in vivo* and the *in vitro* data (Figs. 7 and 8) demonstrate the ability of the A34-containing tRNA to support amino acylation also argues for it to be functional in cytoplasmic translation.

The wobble rules establish that inosine at position 34 may decode codons ending in A, C, or U. Inosine in $tRNA^{Thr}$ may thus be sufficient to decode both the ACU and the ACC codons by wobbling. Why then do these cells keep a lower but significant number of A34-containing tRNAs? We suggest that in the trypanosomatid system, the I34-containing tRNA cannot readily wobble with a U-ending codon. Therefore both tRNAs are dedicated to the decoding of one specific codon, where the ACC codon is decoded by the double-edited and ACU codon by the unedited tRNA, respectively. In addition, recent evidence supports the view that posttranscriptional modifications play an essential role in achieving tRNA functional uniformity helping offset differences among various aminoacyl-tRNAs regarding their binding to the ribosome (34). We could also envisage a situation in which C to U32 editing is not only required for inosine formation *in vivo* but also enhances translational efficiency by providing the necessary changes for structural tRNA uniformity during translation. However, an *in vitro* translation system is not currently available for trypanosomatids, and answering these important questions will thus await further experimentation.

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C to U Editing Stimulates A to I Editing in the Anticodon Loop of a Cytoplasmic Threonyl tRNA in *Trypanosoma brucei*

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