Unusual Noncanonical Intron Editing Is Important for tRNA Splicing in *Trypanosoma brucei*

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SUMMARY

In cells, tRNAs are synthesized as precursor molecules bearing extra sequences at their 5′ and 3′ ends. Some tRNAs also contain introns, which, in archaea and eukaryotes, are cleaved by an evolutionarily conserved endonuclease complex that generates fully functional mature tRNAs. In addition, tRNAs undergo numerous posttranscriptional nucleotide chemical modifications. In *Trypanosoma brucei*, the single intron-containing tRNA (tRNA$^{Tyr}_{GUA}$) is responsible for decoding all tyrosine codons; therefore, intron removal is essential for viability. Using molecular and biochemical approaches, we show the presence of several noncanonical editing events, within the intron of pre-tRNA$^{Tyr}_{GUA}$, involving guanosine-to-adenosine transitions (G to A) and an adenosine-to-uridine transversion (A to U). The RNA editing described here is required for proper processing of the intron, establishing the functional significance of noncanonical editing with implications for tRNA processing in the deeply divergent kinetoplastid lineage and eukaryotes in general.

INTRODUCTION

Critical to the role of tRNAs in protein synthesis are a series of processing events that ensure their proper structure and function. In most eukarya, tRNAs are transcribed in the nucleus as precursor molecules that contain extra sequences: a 5′ leader, a 3′ trailer, and, in fewer cases, introns. Removal of these extra sequences requires a number of enzymes, including RNase P for cleavage of the 5′ leader sequence, RNase Z, and other endonucleases and exonucleases for 3′ end maturation (Mayer et al., 2000). Finally, a specialized tRNA-specific multi-protein splicing machinery removes the introns (Fan et al., 1998). A nontemplated universally conserved CCA tail is also added at the 3′ end of the tRNA by a CCA nucleotidyl transferase. Following nuclear maturation, the tRNA is then exported into the cytoplasm where it can be used for aminoacylation and therefore translation (Phizicky, 2005; Rubio and Hopper, 2011; Wolin and Matera, 1999).

Although 5′ and 3′ end trimming is highly conserved, mechanistically, intron removal varies. In bacteria, tRNA introns are autocatalytic and control their own removal (Reinhold-Hurek and Shub, 1992). In archaea and eukarya, tRNA splicing is initiated by a protein endonuclease that recognizes and cleaves the intron, generating tRNA half molecules that are then joined by a tRNA splicing ligase (Phizicky and Hopper, 2010). In eukarya, despite variations in the number of intron-containing tRNAs and their respective intron sizes, two conserved features exist: (1) tRNA^{Tyr} contains an intron in almost all sequenced eukaryotic genomes (Chan and Lowe, 2009); and (2) most, if not all, introns interrupt the anticodon loop one nucleotide 3′ of the anticodon (Chan and Lowe, 2009). The former underscores an important, but not yet well understood, aspect of tRNA intron maintenance and evolution; the latter implies that intron removal is essential for eukaryotic viability.

At various points during maturation, tRNAs also undergo numerous posttranscriptional chemical modifications placed on the sugar or, at various positions of the base, produce a variety of nucleotides, each with slightly different chemical characteristics. To date, there are more than 100 different modified nucleotides found in tRNAs (Machnicka et al., 2013), and, despite much progress on the role of some modifications in tRNA function, knowledge of the activity and mechanism of most modification enzymes in many organisms is far from complete.

In eukarya, a subset of posttranscriptional changes known as RNA editing may target noncoding RNAs and mRNAs. Editing alters genetic information at the RNA level beyond what can be found in the encoding genes and, as such, can increase genetic diversity. In tRNAs, the most common editing mechanism involves base deamination: "programmed changes" of one canonical nucleotide for another that may impact both their overall structure and function. One type of deamination involves the conversion of adenosine (A) to inosine (I) and has been observed in archaea, bacteria, and eukarya. Additionally, tRNAs may also undergo cytosine (C) to uridine (U) editing, which has been described in archaea, marsupials, kinetoplastids, and plant organelles (Alfonzo et al., 1999; Fey et al., 2001; Janke and Pääbo, 1993). The function of C to U editing of tRNAs varies depending on the position of the edited base in the tRNA. For example, C to U editing can fix stems or restore tertiary base pairing at positions where a nucleotide mismatch is genomically encoded, thus ensuring proper folding (Binder et al., 1994; Maréchal-Drouard et al., 1993). In other instances, C to U editing
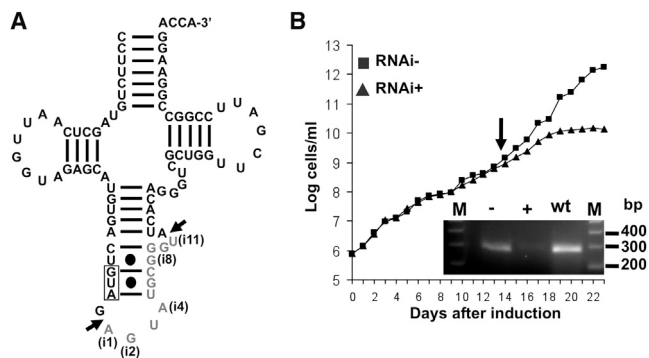
Figure 1. The Putative SEN34 Homolog Is Essential in *T. brucei*

(A) The cloverleaf structure of the single copy intron-containing tRNA, tRNA^{Tyr}, the only intron-containing tRNA in *T. brucei*, is shown. The 11 nucleotide intron is shown in gray letters. The anticodon sequence is boxed. Arrows denote the 5′ and 3′ cleavage sites. Nucleotide positions in the intron are denoted as "i" followed by a number, only the first (i1), last (i11), and edited intron positions (i2, i4, and i8) are highlighted.

(B) A growth curve of tetracycline-induced RNA interference (RNAi+, black triangles) of TbSEN (endonuclease subunit) as compared to an uninduced control (RNAi-, black squares) is shown. The y axis shows a log-scale of cumulative cell densities accounting for total dilutions, and the x axis shows the progression of the growth curve in days. The inset shows semiquantitative RT-PCR with RNA isolated at the onset of the growth phenotype (day 14, arrow) from RNA⁺ (+), RNAi⁻ (−), and wild-type (wt) cells. M refers to a 100 bp size marker (Invitrogen); the region between 200 and 400 bp is shown. See also Figure S1.

(analogous to A to I editing) affects the anticodon, changing the decoding ability from one codon to another and effectively expanding the decoding properties of the edited tRNA (Alfonzo et al., 1999).

In total, the mechanisms that trim, splice, modify, and edit tRNA molecules are required for the proper folding, recognition, and function of mature tRNAs in translation. In the present study, we are focusing on the identification of all the components of the *Trypanosoma brucei* tRNA splicing endonuclease. Speaking to its degree of divergence, we could only identify a single putative subunit of the four highly conserved subunits of other eukaryotes. Here, the role of this subunit in tRNA splicing is demonstrated by RNA interference. Surprisingly, analysis of the tRNA intron sequence led to the discovery of a number of noncanonical RNA editing events, and these are shown to be important for tRNA splicing. Sequence comparisons presented here also show various degrees of representation of the edited introns in the genomes of trypanosomes, highlighting distinct evolutionary paths for the occurrence of such unusual noncanonical editing events.

RESULTS

The Putative *T. brucei* SEN34 Is Essential for Splicing and Viability

Little is known about tRNA splicing in early divergent eukaryotes, such as kinetoplastids, which include *Leishmania* and *Trypanosoma*, responsible for devastating diseases worldwide. In *T. brucei*, only tRNA^{Tyr}, a single-copy gene, contains an intron

(Mottram et al., 1991; Schneider et al., 1993) (Figure 1A). Bioinformatic analysis of the kinetoplastid genomic databases (TriTrypDB), with various subunits of the yeast and human tRNA splicing endonuclease (SEN2, SEN34, SEN54, and SEN15) (Paushkin et al., 2004; Trotta et al., 1997) revealed the presence of a potential SEN homolog in *T. brucei* (TbSEN) (Figure S1 available online). To investigate the role of TbSEN in tRNA splicing in *T. brucei*, its protein coding region was cloned into a *T. brucei* tetracycline-inducible RNAi vector (Wickstead et al., 2002). Following RNAi induction by tetracycline, we observed a growth phenotype and eventually cell death; thus, TbSEN is essential for growth (Figure 1B). A substantial decrease of the putative TbSEN mRNA was also observed by RT-PCR analysis with TbSEN-specific oligonucleotide primers (Figure 1B, graph inset). These results are consistent with those reported in a high-throughput RNAi screen coupled to parallel sequencing, which showed that downregulation of the same gene led to a significant decrease in *T. brucei* fitness, although these authors did not assign a function to the gene (Alsford et al., 2011).

The Only tRNA Intron of *T. brucei* Undergoes Noncanonical Editing

To determine the level of intron-containing tRNA^{Tyr} in these cells, northern hybridization experiments were performed using an intron-specific probe and total RNA fractions from RNAi-induced and RNAi-uninduced cells. Downregulation of TbSEN should lead to accumulation of the intron-containing tRNA. Surprisingly, we were unable to detect a significant hybridization signal using this probe in both RNA fractions (Figure 2A).

Possibly in uninduced cells, intron-containing tRNA^{Tyr} is a short-lived intermediate that escapes detection by northern analysis. Thus, we took a more sensitive approach using RT-PCR with the same RNA fractions and oligonucleotide primers specific for the exons of tRNA^{Tyr}. Downregulation of TbSEN leads to the appearance of a slower migrating band on nondenaturing gels, consistent with accumulation of the intron-containing species (Figure 2B). This species also partially accumulated in the uninduced cells, likely due to the well-established leakiness of the *T. brucei* RNAi system (Kolev et al., 2011; Ullu et al., 2004). We cloned this fragment into a plasmid vector and sequenced plasmid DNA from 25 independent clones. None of the clones matched the genomic sequence; instead, we found two populations of intron sequences: one containing G to A and A to U alterations in the intron (16 out of 25 clones or 64%) and the other with an additional G to A change (9 out of 25 clones or 36%) (Figure 2C). Similar experiments with total DNA isolated from wild-type cells showed that all clones (a total of 25 independent clones) contained sequences identical to those reported in the *T. brucei* genome database (TriTrypDB) (data not shown). The data are consistent with the alteration of nucleotides in the sequence of the single tRNA intron occurring in at least two of the three sites with >96% efficiency (where 1 of 25 clones would represent a theoretical value for unedited sequences of 4%).

To further substantiate tRNA^{Tyr} intron editing, we probed the same northern blot as before with intron-specific probes that include the editing changes. These could now detect the intron-containing tRNAs (Figure S2). This demonstrates that the observed nucleotide changes are not the result of either
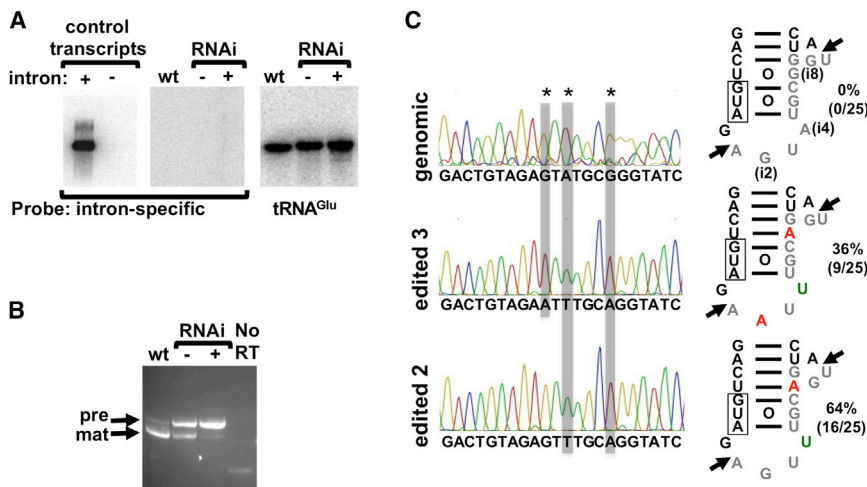
Figure 2. The Intron of tRNA^{Tyr} Undergoes Noncanonical Editing

(A) Control RNA transcripts and RNA extracted from the RNAi induced, uninduced, and wild-type cells (as above) analyzed by northern hybridization with an intron-specific probe. The "control transcripts" (left) refer to in vitro-transcribed control tRNAs that either contain (+) or lack (−) the intron used as positive and negative controls for intron detection. RNA extracted from the RNAi induced, uninduced, and wild-type cells (as above) were transferred to a separate membrane and probe with the same probe (center). The same membrane was stripped and rehybridized with a probe specific for tRNA^{Glu} used as a positive control for hybridization (right).

(B) RT-PCR experiments with the same RNA samples as before but with oligonucleotide primers specific for the exons of tRNA^{Tyr}, which do not discriminate between mature (mat) and intron-containing tRNA (pre), shown by arrows. No RT refers to a mock PCR reaction in the absence of reverse transcriptase used to control for DNA contamination in these samples.

(C) The "pre" band from the gel above was excised, the purified DNA was cloned into plasmids, and 25 independent clones were analyzed by automated sequencing. Representative sequencing traces are shown; positions of noncanonical editing are marked by an asterisk and highlighted by gray boxes. The schematics on the right show the anticodon stem portion of tRNA^{Tyr} with the intron in gray letters and the observed noncanonical edits, G to A (green letters) and A to U (red). Percentages denote the number of clones of each type observed divided by the number of total cones sequenced (in parentheses) × 100. The arrow marks the cleavage sites (as above). The data are representative of at least four independent experiments for each section. The edited nucleotide positions in the intron are labeled as above (i2, i4, and i8).

See also Figure S2.

RT-PCR or sequencing artifacts. Interestingly, we observed higher levels with the probe specific for the tRNA edited at two positions (Figure S2, edited 2) compared to the other species (Figure S2, edited 3). These differences in the relative levels of the two edited species are consistent with our sequencing data.

In an attempt to further assess editing levels, RNA isolated from RNAi-induced and RNAi-uninduced cells were used in "poisoned" primer extension assays (Alfonzo et al., 1999) where editing would lead to incorporation of the chain terminator dideoxyTTP (ddTTP) (Figure 3A). Again, with this approach, we could detect a "strong" stop at the position corresponding to the primer extended by two nucleotides, which was indicative of editing (Figure 3B, left panel). The only significant additional stop corresponded to a signal at primer plus 3 nucleotides, but this signal is due to mispriming via the mature tRNA sequence (Figure 3B, right panel, mature control transcript). Mispriming may occur because of the sequence similarity between the primer annealing sites for the precursor tRNA (intron containing) and the mature sequence (Figure S3). In addition, several fainter bands were observed with nuclear and cytosolic RNA fractions from wild-type cells (Figure 3B, right panel). These read-through products are visible because of incomplete termination with ddTTP, but their sizes corresponded to primer plus 6 nucleotides (i4 position of the intron) and primer plus 8 nucleotides, the 5′-most editing site (i2 position of the intron), consistent with unedited and edit 3 transcripts, respectively. The low-level signal with these additional bands is in agreement with the high editing efficiency observed by RT-PCR sequencing.

It is possible that the observed noncanonical changes are due to the general poor physiological state of the cells following RNAi of an essential gene. We also isolated nuclear RNA from wild-type cells to determine (1) if editing occurs in wild-type cells and (2) where editing is localized within cells. In nuclear RNA fractions, we detected intron-containing tRNA edited to similar levels as the tRNA isolated from the RNAi strain (Figure 3B, right panel). These results support the view that the noncanonical editing described here occurs naturally in the nucleus of T. brucei. Edited tRNA was also detected in the cytoplasmic fractions, likely due to breakage of the nuclei during preparation, which leads to nuclear contamination of the cytoplasmic fractions as shown by significant hybridization of the cytoplasmic fractions with a probe specific for U6 snRNA (a nuclear marker) (Figure S4).

Because of the prevalence of posttranscriptional modifications in tRNAs, it is possible that the observed editing changes are due to some unusual modification, which leads to misreading by the reverse transcriptase. To explore this possibility, we purified native intron-containing tRNA^{Tyr} and mature tRNA^{Tyr} from the RNAi cell line (following induction by tetracycline). Total RNA from these cells was hybridized to a biotinylated oligonucleotide (as described previously) (Alfonzo et al., 1999). This oligonucleotide does not discriminate between the intron-containing tRNA and the mature tRNA. Following hybridization, the tRNAs were gel purified and subjected to postlabeling (Alfonzo et al., 1999). Although this approach does not reveal the specific location of modifications, it shows the total modification set for each species. We found that the intron-containing tRNA had negligible levels of modifications, whereas the mature was fully modified (Figure 4). Importantly, some of the modifications detected in the mature tRNA (for example, acp^{3}U and m^{7}G) occur only once per tRNA molecule (Machnicka et al., 2013), arguing that the lack of modifications in the intron-containing tRNA is not
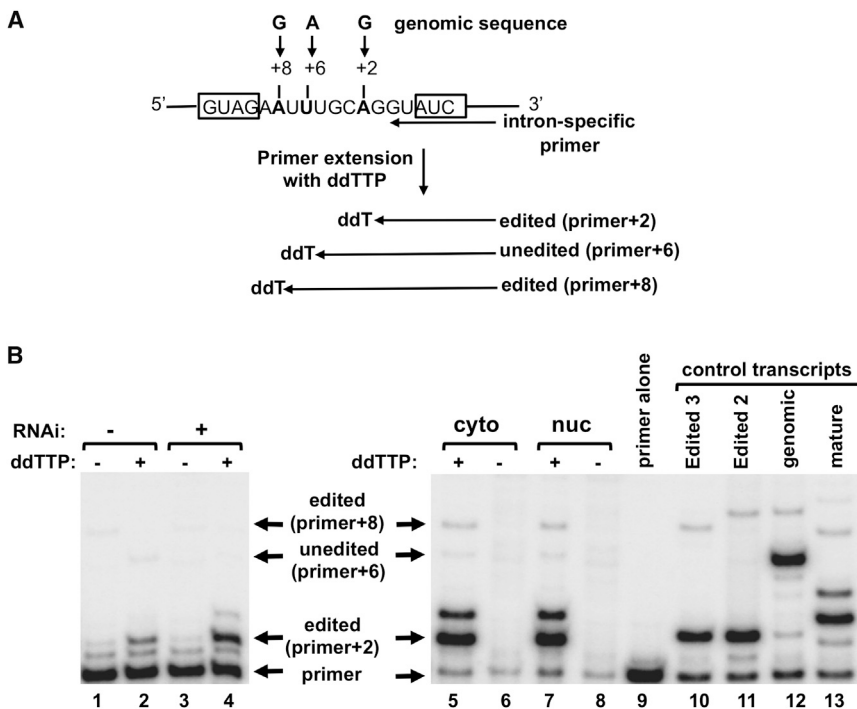
Figure 3. Efficient Noncanonical Editing Is Localized in the Nucleus

(A) Diagram describing the "poisoned" primer extension assay. A radioactive intron-specific primer that anneals two nucleotides 3′ of the editing site is shown (intron-specific primer). The reaction was performed in the presence of the chain terminator ddTTP (ddT), which, if incorporated at the edited position, yields a primer plus two-nucleotide product. A primer plus six nucleotides is indicative of a lack of editing at the 3′-most editing site (G to A). Editing sites are indicated by arrows, and the exonic sequences are boxed. The edited positions are shown in bold letters. +8, +6, and +2 indicate the distance in nucleotides of each edited position from the last nucleotide of the primer.

(B) "Poisoned" primer extension reactions of total RNA isolated from the RNAi uninduced (−, lanes 1 and 2) and induced (+, lanes 3 and 4) cells, as before. Reactions were performed in the absence (−, lanes 1 and 3) or presence (+, lanes 2 and 4) of ddNTP (left). The right panel shows similar reactions as in the left, but performed with subcellular RNA fractions, nuclear (nuc) and cytoplasmic (cyto), isolated from wild-type cells. The arrows denote the position expected for edited (primer + 2 and primer + 8) and unedited (primer + 6) products in these reactions as well as the primer. Control transcripts refer to RNA generated in vitro and used as markers for the different expected products (as indicated). Mature refers to an in vitro generated intronless transcript used as a control for mispriming during the assay. See also Figures S3 and S4.

due to the modifications existing at such low levels that they escape detection. This experiment suggests that (1) in contrast to other systems, there are no intron-dependent modifications and (2) no unusual modifications could account for the editing changes, which are likely due to replacement by canonical nucleotides.

Noncanonical Editing Is Important for tRNA^Tyr Splicing

We next investigated the possibility that editing of the intron plays a role in tRNA splicing. We partly purified the endonuclease from *T. brucei* by following previously published detailed protocols for the enrichment of endonuclease activity from yeast (Peebles et al., 1983). We tested the efficiency of cleavage by this enriched *T. brucei* fraction on substrates site-specifically labeled at the first position of the intron (i1, the 5′-most nucleotide of the intron). Three different substrates were tested: either the two edited transcripts or an unedited version of the intron (representing the genomic sequence). The edited pre-tRNAs were efficiently cleaved as compared to the unedited pre-tRNAs (Figures 5A and 5B). Furthermore, we observed transfer of the label to the 3′-most nucleotide of the 5′ exon, allowing visualization of the free 5′ exon. This is consistent with the cleavage chemistry observed for other tRNA splicing endonucleases regardless of the organism (Abelson et al., 1998). This observation is also in line with previous work in *T. brucei*, showing that, in vitro, a single nucleotide mutation at the first position of the anticodon abrogates splicing (Schneider et al., 1993). Although this position is not precisely the 3′-most edited position

described here, it would disrupt a canonical base pair between the anticodon and the intronic sequence. Taken together, these results suggest the importance of a specific structural requirement formed by editing of nucleotides to create a fully base-paired intron stem in the *T. brucei* system.

We then constructed an intron-containing tRNA variant in which two base pairs of the anticodon stem were flipped (Figure 6A, schematic). This construct could allow similar RT-PCR analysis as before, while differentiating this variant from the endogenous wild-type tRNA^Tyr. This construct was integrated in the genome where expression is still driven by the native RNA polymerase III. Total RNA from this strain was used in a reverse transcription reaction performed with an oligonucleotide primer that contained the 3′ tag and overlapped the intronic sequence as to bias the cDNA toward intron/"tag"-containing tRNA (Figure 6B). The PCR reaction was then performed with the "tag"-specific primers, which yielded a product of a size consistent with that of the intron-containing tRNA. This product was analyzed by diagnostic restriction digest for either edited (cleaved by BfuAI) or unedited (cleaved by AciI, but not BfuAI) products. RT-PCR analysis showed that the mutations in this tRNA variant led to complete inhibition of editing (Figure 6B, left panel; compare AciI to BfuAI digests). We do not understand the reason for this, other than to suggest that those positions may be important for recognition by a factor important for editing.

We also performed similar RT-PCR reactions, but we used only the primers specific for the exonic "tags." In this variant,
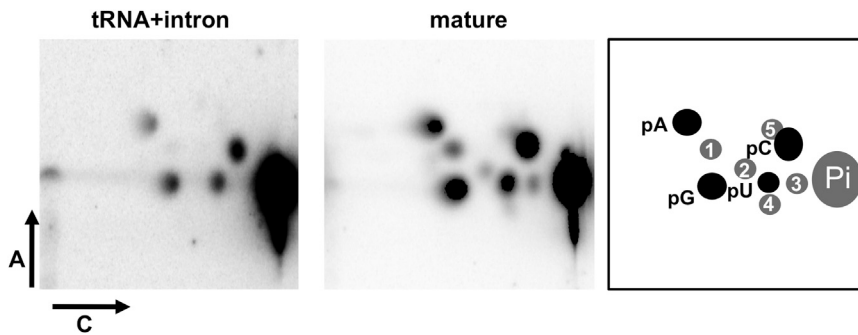
Figure 4. Native Pre-tRNA^Tyr Has Negligible Modification Levels

Two-dimentional thin-layer chromatography (2D-TLC) of native tRNA^Tyr purified from the RNAi-induced cells. The purification procedure does not discriminate between mature and intron-containing tRNA (pre-tRNA). Postlabeling analysis of the two native species shows the presence of modified nucleotides only in the mature tRNA (center panel). These modifications are commonly found in tRNA^Tyr from other eukaryotes and are often found in most tRNAs. The left panel shows the lack of modified nucleotides in the intron-containing tRNA. The right panel shows a key with 5-monophosphorylated nucleotide assignments for the right and center panels. 1, pQ or pG$_m$, queuosine or 2′-O-methylguanosine; 2, pΨ$_m$, 2′-O-methylpseudouridine; 3, acp^{3}U, 3-amino-3-carboxypropyluridine; 4, pΨ, pseudouridine; 5, pm^{7}G, 7-methylguanosine. pA, pC, pG, and pU refer to the unmodified 5′ ribonucleotides. Pi refers to inorganic phosphate released following incubation with P1 nuclease. Arrows labeled A and C represent the two dimensions used during thin-layer chromatography (TLC), as described in Experimental Procedures. All modification assignments are based on published maps (Grosjean et al., 2004, 2007). None of these modifications are known to alter base pairing during reverse transcription.

both editing and splicing were inhibited, suggesting a possible connection between the two events (Figure 6B, center panel). As a control, we generated a tRNA variant with the same sequence "tags," but in which a fully edited intron was provided (Figure 6B, right panel). A significant amount of tagged mature tRNA was observed with this variant, supporting the view that in vivo noncanonical editing is essential for splicing (Figure 6B, compare center and right panels). In this experiment, there is still a portion of the tRNA that remains unspliced, presumably due to the fact that the tag mutations also affect splicing. This last observation suggests that editing and splicing share common recognition motifs (Figure 6B, right panel). However, we cannot rule out the possibility that the accumulated unspliced "tagged" tRNA is simply due to the general overexpression of the tRNA when transcribed from a plasmid.

Evolutionary Conservation of Noncanonical Editing in Kinetoplastids

We also investigated the potential prevalence of intron editing in kinetoplastids in general. In all kinetoplastids, tRNA^Tyr is the only intron-containing tRNA. We aligned the genomic intron sequences from various kinetoplastids (genus *Crithidia*, *Leishmania*, etc.), revealing that the edited nucleotides exist, to various degrees, at the DNA level in these genomes (Figure 7A). Most astonishing is that *L. tarentolae* encodes two copies of tRNA^Tyr in the genome, precisely matching the sequence of each edited species found in *T. brucei* at the RNA level (Figure 7B).

DISCUSSION

In all organisms, introns disrupt the sequence of tRNAs, and their precise removal is essential to generate a mature molecule that can engage in protein synthesis. Archaea and eukarya share a common mechanism for tRNA splicing, which involves a protein endonuclease that cleaves the intron at the two spliced sites and initiates the splicing process (Calvin and Li, 2008; Hopper and Phizicky, 2003; Phizicky and Greer, 1993). All eukaryal tRNA endonucleases described so far (for example, from plants, mam-

mals, and yeast) contain four distinct subunits (Calvin and Li, 2008). Two subunits contain the enzyme's active site required for cleavage (SEN2 and SEN34), yet the other two subunits (SEN15 and SEN54) are essential structural components. Our analysis of the kinetoplastids databases (which include *T. brucei*) revealed one bioinformatically recognizable subunit of the endonuclease, which, despite divergence, can still be assigned as a SEN34 homolog based on sequence comparative analysis (Figure S1). Additionally, the *T. brucei* genome encodes a single intron-containing tRNA. This intron is only 11 nucleotides long, which is close to the lower theoretical limit required for cleavage (Di Nicola Negri et al., 1997; Fabbri et al., 1998; Tocchini-Valentini et al., 1993).

Beyond splicing comes the question of the unusual editing observed in this intron. The RNA editing repertoire includes a number of disparate mechanisms, many of which serve to alter coding capacity. These include uridine insertions and deletions in trypanosomatid mitochondria, C insertions in *Physarum*, nucleotide deaminations (C to U and A to I) in various coding and noncoding RNAs (including tRNAs) (Aphasizhev and Aphasizheva, 2011; Blanc and Davidson, 2010; Chateigner-Boutin and Small, 2011; Gommans, 2012; Göringer et al., 2011; Jackman and Alfonzo, 2013; Jackman et al., 2012; Mallela and Nishikura, 2012; Paris et al., 2012; Sie and Kuchka, 2011; Smith et al., 2012). All of these reactions have been extensively studied, and the various editing activities have been reconstituted in vitro. New sequencing technologies have now uncovered many new editing sites in a number of organisms including humans, the functions of which are not currently clear (Li et al., 2011; Rosenberg et al., 2011; Sakurai et al., 2010). In addition, a number of editing events have been found essential for mRNA splicing (Castandet et al., 2010; Lamattina et al., 1989; Petschek et al., 1996; Rueter et al., 1999). Most recently, using deep sequencing approaches, potentially novel nonconical editing events were described as widespread in the human transcriptome (Li et al., 2011). However, it is still not clear to what extent these events exist. A number of these can be alternatively explained by known difficulties and potential artifacts in interpreting the vast amounts of data derived from these approaches. In the present work, we
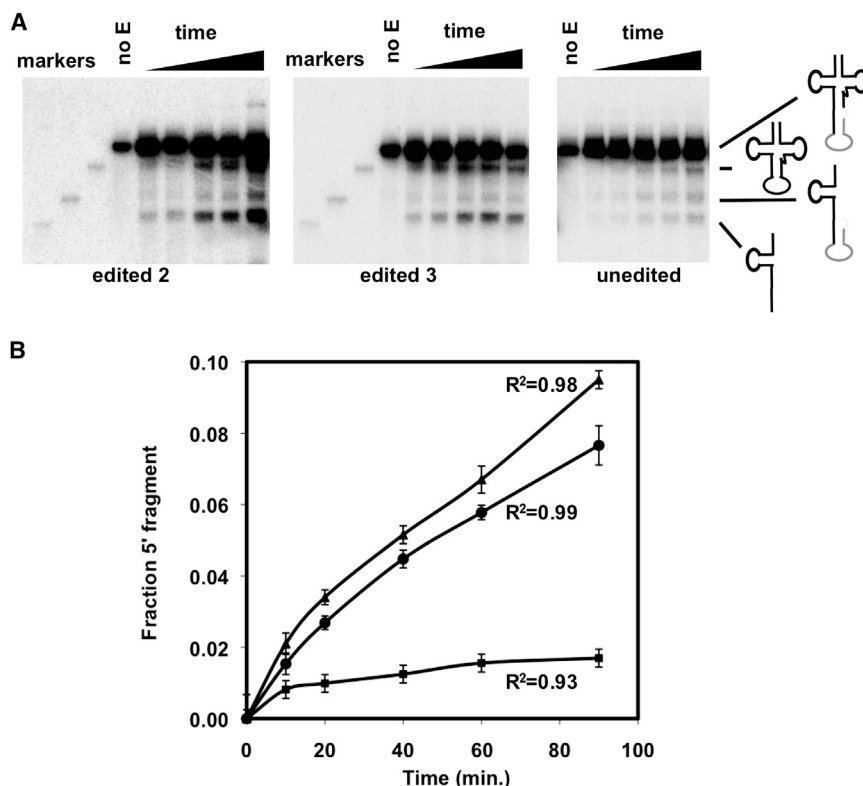
Figure 5. The Edited Intron Is the Preferred Substrate for In Vitro Cleavage

(A) Cleavage assays using an enriched endonuclease fraction from *T. brucei* and chemically synthesized intron-containing tRNA^Tyr corresponding to either an intron with the unedited sequence (genomic) or edited at two (edited 2) and three positions (edited 3) are shown. These substrates were site-specifically labeled at the 5′-most nucleotide of the intron in the pre-tRNA and incubated with a constant and subsaturating concentration of enzyme fraction at increasing times. The size markers on the left (also shown pictorially on the right) correspond to mature (no intron), 5′ exon-intron intermediate and the mature 5′ exon.

(B) Progress curves of the cleavage reactions on (a), where the tRNA with the unedited intron is denoted by black squares and the tRNA substrates with the edited introns are shown by black triangles (edited 2) and black circles (edited 3). Each curve is the average of three independent assays and their R^2 values are as indicated. Error bars represent the standard error from the mean.

demonstrate that the noncanonical editing described here is required for processing of a tRNA intron, which is in turn essential for viability. We have presented several lines of evidence using more classical sequencing and molecular approaches to substantiate our observations. Taken together, our data support the view that noncanonical editing occurs in at least one tRNA. Still outstanding is the nature of the editing enzyme. Currently, no enzymatic activity has been described that could account for these noncanonical editing changes. Mechanistically, no deamination event could be at play, and the activity likely involves either nucleotide or base replacement. Our attempts to recreate this editing activity in vitro has yet to provide conclusive answers. Regardless, it is difficult to imagine that such an activity is only relegated to editing a single intron in kinetoplastids and likely has additional cellular targets.

An interesting question is why kinetoplastids still maintain an intron in a single tRNA. This fact points at some secondary function that has led to selection for, and maintenance of, the intron. Again, one reason may rest on previous work, where the presence of the intron is required for the modification of the anticodon (Grosjean et al., 1997; Johnson and Abelson, 1983; Szweykowska-Kulinska et al., 1994). This does not seem to be the case with *T. brucei*, given that no modifications were observed in the native intron-containing tRNA, with the exception of the edited positions shown in this work. It is also possible that the intron itself may play some chaperoning role in ensuring proper L-shape folding of the tRNA: an essential structure for splicing in eukaryotes (Calvin and Li, 2008). In this realm, intron editing may provide order to the folding process

while avoiding formation of nonfunctional undesirable alternative conformers.

The pivotal observation of both edited intron populations encoded in the genome of *L. tarentolae* is significant in at least two respects: (1) it indicates that this type of editing may occur in other kinetoplastids and (2) unlike *T. brucei*, intron editing may not be required in all kinetoplastids. However, it is apparent that the involvement of editing as an important splicing determinant is a naturally selected trait. As to what extent it appears in other organisms, only time will tell. Clearly, the possibility of altering RNAs to this degree by noncanonical editing opens a realm of possibilities and contributes greatly toward pushing the limits of the editing field and RNA biology in general.

EXPERIMENTAL PROCEDURES

RNAi Induction and Cell Cultivation

A 624-nucleotide-long portion of the putative TbSEN34 gene (Tb927_11_v5) was PCR amplified from the total genomic DNA of *T. brucei* strain 29-13. The amplicon was cloned into the p2T7-177 vector, which was linearized with Not I (for genome integration) and then used to transform procyclic *T. brucei* 29-13 cells, and clonal lines were selected as described elsewhere (Wickstead et al., 2002). RNAi was triggered by the addition of 1 μg/ml of tetracycline to the growth medium (SDM-79). Cell density was measured every 24 hr using the Beckman Z2 Coulter counter over a period of 22 days after the induction of double-stranded RNA synthesis.

Endonuclease Cleavage Assay

RNA halves were commercially synthesized; these corresponded to either the two edited pre-tRNAs (containing the intron) or a version corresponding to the genomic sequence (unedited). The RNA pieces were designed so that the 5′-most nucleotide of the 3′ fragment corresponded precisely to that of the first nucleotide of the intron. This fragment was radioactively end labeled as previously described and joined to the 5′ fragment by splint ligation (Moore
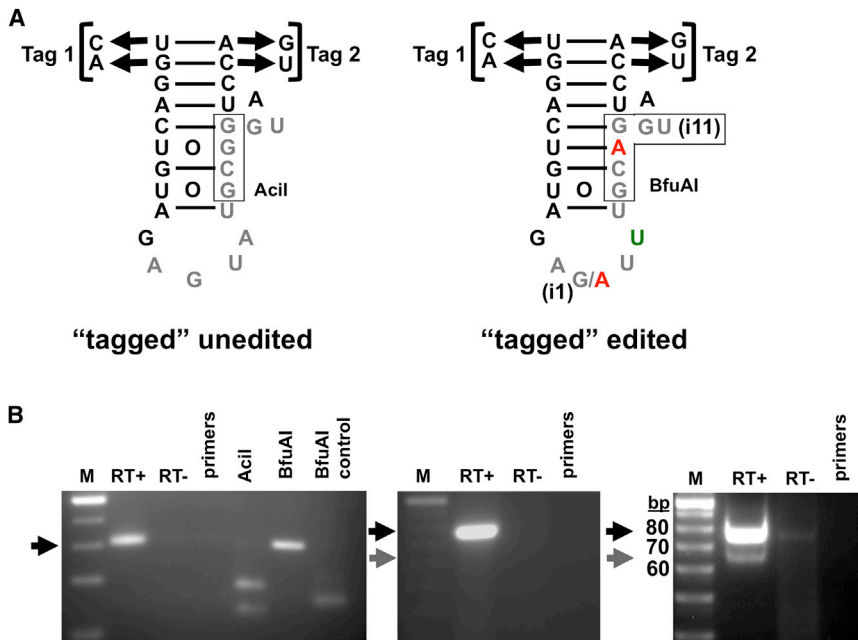
Figure 6. Editing Is Important for tRNA Splicing

(A) Schematic diagram of the expected products for the intron-containing tRNA "tagged" variant generated by flipping the two base pairs of the anticodon stem of tRNA[Tyr] (as indicated) producing two complementary tags (Tag1 and Tag2). The sequence of the intron corresponding to the unedited tRNA contains an AciI restriction site, which, upon editing, becomes a BfuAI site. These restriction sites (boxed) were used as diagnostic for one or the other product during RT-PCR reactions specific for the tagged constructs (below). (B) RT-PCR reactions of only the "tagged" intron-containing tRNA variant (left panel). An oligonucleotide primer specific for the intron and that contains the Tag2 sequence was used to generate a cDNA specific for intron-containing tagged species. Two additional primers whose 3′ ends were anchored at the tags were then used for the PCR reaction. Reactions were performed in the presence (RT+) or absence (RT−) of reverse transcriptase, where the RT− reaction serves as a DNA contamination control. The products from the RT+ reaction were digested with either AciI, which only cleaves a DNA product corresponding to the unedited intron, or BfuAI, which only cleaves the edited species but does not discriminate between the edited two and three species. A synthetic edited transcript was also used, and the resulting RT-PCR product was digested with BfuAI as a positive control for digestion (BfuAI control) (left panel). The center panel shows an RT-PCR reaction with the tag-specific oligonucleotide primers, which do not discriminate between the mature and intron-containing tagged tRNAs. A full-length intron-containing product, shown by the black arrow (76 bp expected size), demonstrating the accumulation of the unspliced tRNA is shown. The gray arrow denotes the expected position for the mature tagged tRNA product (65 bp expected size). The right panel shows a similar assay with a tagged construct containing a fully edited intron, showing that this variant is proficient in splicing. "M" refers to a 10-bp size marker (Invitrogen) and "bp" indicates size in base pairs.

and Sharp, 1992). This generated the site-specifically labeled substrates used in the cleavage assays. These substrates were incubated in yeast endonuclease cleavage buffer (Peebles et al., 1983) with a partially purified *T. brucei* endonuclease fraction generated by sequential column chromatography using the same protocol used to enrich the yeast enzyme (Peebles et al., 1983). The tRNA substrates were incubated for various times under conditions where the substrate was saturating. Following incubation, the resulting products were extracted with Tris-buffered phenol and precipitated by the addition of 0.3 M sodium acetate (pH 4.5) and 3 vol of 100% ethanol followed by centrifugation at 13,000 RPM. The resulting pellets were resuspended in denaturing urea buffer and separated under denaturing conditions in a 10% acrylamide/7 M urea gel. Following electrophoresis, the gel was dried and the reaction products visualized and quantitated with a Storm PhosphoImager (Molecular Dynamics).

Northern Blot Analysis and Primer Extension Assay

RNA was isolated using the guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). Ten micrograms of total *T. brucei* RNA were separated on denaturing 8% polyacrylamide gel with 7 M urea and electroblotted to Zeta-probe (Bio-Rad) membranes, which were subsequently probed with [32]P-5′ end-labeled oligonucleotide specific for each RNA (5′-TGATACCCGCATACTCTAC-3′ for genomic, 5′-TGATACCTGCAAA CTCTAC-3′ for edited 2, and 5′-TGATACCTGCAAATTCTAC-3′ for edited 3). Hybridization procedures were carried out according to the manufacturer's instructions (Bio-Rad). Images were taken with a Storm PhosphorImager (Molecular Dynamics).

Poisoned primer extension analysis was carried out as described previously (Alfonzo et al., 1999). Briefly, a [32]P-5′-labeled oligonucleotide primer complementary to the pre-tRNA and anchored at the intron (5′-TCGAACCAGC GACCCTGTGATAC-3′) was used in reactions containing dATP, dGTP, and dCTP and the chain terminator ddTTP in place of dTTP. Control reactions were also performed with all four standard nucleotides; these were used as

controls to ensure that any "strong" stops seen in the "poisoned" primer extension reactions were not due to the secondary structure of the tRNA. In vitro transcripts corresponding to the different edited and unedited species as well as mature tRNA[Tyr] were also used in control reactions and also served as size markers.

RT- PCR and Sequencing

Total RNA from *T. brucei* (5 μg) was used for RT-PCR reactions as described by the manufacturer (Invitrogen). Briefly, RNA samples were heated at 70°C for 10 min in the presence of 2 pmol of a reverse oligonucleotide primer (5′-AATC GAACCAGCGACCCTGTGA-3′) complementary to the 3′ exon sequence of wild-type tRNA. This primer does not discriminate between mature and intron-containing tRNA[Tyr]. This was followed by addition of cDNA reverse transcription buffer and deoxynucleotides to a final concentration of 0.5 mM. The reaction was incubated at 42°C for 2 min, at which point 200 U of Superscript II reverse transcriptase (Invitrogen) was added and then further incubated for 50 min at 42°C. A similar reaction incubated in the absence of reverse transcriptase was used as a negative control (RT- control) and also as a control for DNA contamination of the RNA fraction. The resulting cDNA was PCR amplified as described previously (Alfonzo et al., 1999) using the same reverse oligonucleotide primer as above and a second primer (5′-CCTTCTGTAGCT CAATTGGTAG-3′) specific for the 5′ exon. The resulting PCR products were separated on a 10% nondenaturing polyacrylamide gel, and the intron-containing band was excised and eluted. This product was then cloned into a pCRII-TOPO (Invitrogen) plasmid vector, transformed into *E. coli*, and 25 independent clones were sequenced by automated sequencing (ABI-Life Technologies). Reactions omitting the reverse transcription step were performed with total DNA isolated from *T. brucei* and the same PCR primers. Likewise, 25 independent clones were sequenced and used as the genomic control.

Similar reactions with oligonucleotides specific for the tagged sequence were used to analyze both editing and splicing using the tagged tRNA variants described in Figure 6. For this reaction, the reverse transcription step was
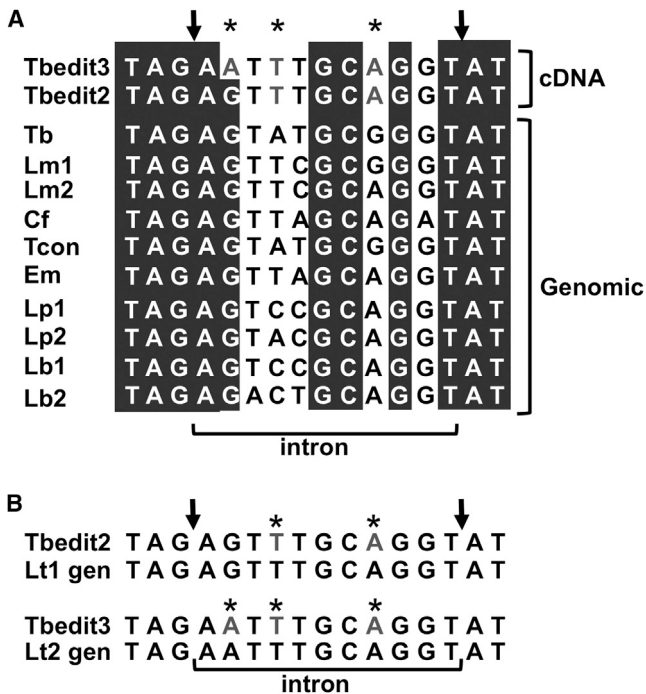
Figure 7. Evolutionary Conservation of the Edited Introns in Kineto-plastids

(A) Sequence alignment of the intron portion of tRNA^{Tyr} from various kinetoplastids showing potential editing in closely related organisms.

(B) The closely related kinetoplastid *Leishmania tarentolae* encodes two copies of tRNA^{Tyr} with intron sequences matching precisely those observed in *T. brucei* at the RNA level. The arrows mark the cleavage sites and the asterisks denote those positions found to undergo noncanonical editing in *T. brucei*. "cDNA" refers to the two edited sequences obtained from *T. brucei*. Genomic refers to sequences obtained from the genomic database (TriTryp). Tb, *Trypanosoma brucei*; Lm, *Leishmania major*; Cf, *Crithidia fasciculata*; Tcon, *Trypanosoma congolense*; Em, *Endotrypanum monterogeii*; Lp, *Leishmania panamemsis*; Lb, *Leishmania braziliensis*.

performed with an oligonucleotide anchored at the intron but that contained the Tag2 sequence (5′-TCGAACCAGCGACCCCATGATAC-3′, tag nucleotides are underlined). The resulting cDNA was used in a PCR reaction with oligonucleotide primers that extend the length of the tRNA and which are anchored at the Tag1 and Tag2 sequences (Figure 6A).

Thin-Layer Chromatography Analysis

Native and intron-containing tRNAs were purified from total RNA isolated from Tb-SEN RNAi-induced cells following 15 days of induction. The tRNAs were purified using an antisense biotinylated oligonucleotide (5′-CCTTCCG GCCGGAATCGAACCAGCGACCCTG-3′) and streptavidin beads (Sigma) using a procedure described previously (Alfonzo et al., 1999; Crain et al., 2002). This oligonucleotide does not discriminate between mature and pre-tRNA (intron containing). The resulting products were isolated by purification on a 7 M urea-10% acrylamide gel. The gel-purified tRNAs were separately de-phosphorylated with calf intestinal phosphatase (Invitrogen) and digested with RNase T2 for 5 hr at 37°C in 10 mM ammonium acetate buffer (pH 4.5). The resulting 3′ nucleotide monophosphates were labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP for 45 min at 37°C in the appropriate buffer (Invitrogen). To remove unincorporated radioactive ATP, the mixture was treated with 5 U of apyrase (Sigma), and unlabeled ATP was added to a final concentration of 1 mM. The mixture was incubated further at 37°C for 2 hr. This treatment yields a mixture of 5′-labeled 5′,3′ phosphorylated nucleotides. Following the apyrase treatment, the samples were treated with nuclease P1 (5 U/10 l reac-

tion) 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 cpm were loaded onto a cellulose thin-layer chromatography plate (Merck) and analyzed by 2D-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 4). 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). After chromatography, the plates were dried at room temperature and subjected to PhosphorImager analysis (Molecular Dynamics). Nucleotide assignments were made using published maps (Grosjean et al., 2007; Grosjean et al., 2004).

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