Cell, Vol. 108, 637–648, March 8, 2002, Copyright ©2002 by Cell Press

Trypanosome Mitochondrial 3′ Terminal Uridylyl Transferase (TUTase): The Key Enzyme in U-Insertion/Deletion RNA Editing

Ruslan Aphasizhev,[1] Sandro Sbicego,[2,6] Marian Peris,[4,6] Sei-Heon Jang,[3] Inna Aphasizheva,[2] Agda M. Simpson,[1] Anatoly Rivlin,[2] and Larry Simpson[1,2,5]

[1]Department of Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles
Los Angeles, California 90095
[2]Howard Hughes Medical Institute
University of California, Los Angeles
Los Angeles, California 90095
[3]Taegu University
Department of Molecular Biology
Kyung-San
Kyung-Pook 712-714
South Korea
[4]Molecular and Cell Biology Department and
Howard Hughes Medical Institute
University of California, Berkeley
Berkeley, California 94720

Summary

A 3′ terminal RNA uridylyltransferase was purified from mitochondria of *Leishmania tarentolae* and the gene cloned and expressed from this species and from *Trypanosoma brucei*. The enzyme is specific for 3′ U-addition in the presence of Mg^{2+}. TUTase is present in vivo in at least two stable configurations: one contains a ~500 kDa TUTase oligomer and the other a ~700 kDa TUTase complex. Anti-TUTase antiserum specifically coprecipitates a small portion of the p45 and p50 RNA ligases and approximately 40% of the guide RNAs. Inhibition of TUTase expression in procyclic *T. brucei* by RNAi downregulates RNA editing and appears to affect parasite viability.

Introduction

Uridine (U) insertion/deletion RNA editing of mRNA transcripts in trypanosomatid protozoa involves a series of enzymatic reactions mediated by base-pairing with guide RNAs (gRNAs) (Estévez and Simpson, 1999). The model involves the initial formation of an anchor duplex by the cognate gRNA just downstream of the editing site, a cleavage of the preedited mRNA at the first mismatch, and the addition of Us to the 3′ end of the 5′ cleavage fragment by a 3′ uridylyl transferase (TUTase), which base pair with the guiding nucleotides in the gRNA. The two cleavage fragments are then ligated by an RNA ligase (Rusché et al., 2001; Schnaufer et al., 2001; McManus et al., 2001).

TUTase activities have been partially purified from mammalian cells (Andrews and Baltimore, 1986; Milchev and Hadjiolov, 1978) and plants (Zabel et al., 1981). A 3′ TUTase activity specific for the addition of Us to the mammalian U6 snRNA prior to maturation has been identified (Trippe et al., 1998). A 3′ TUTase activity in trypanosomes was first described in whole-cell extracts of *T. brucei* (White and Borst, 1987) and in a purified mitochondrial fraction from *L. tarentolae* (Bakalara et al., 1989).

In this paper, we describe the isolation and characterization of trypanosomatid TUTase and its interaction with other editing activities. We also present biochemical and genetic evidence for an involvement of this enzyme in U-insertion RNA editing.

Results

Isolation of 3′ TUTase from *L. tarentolae* Mitochondria

A 3′ TUTase activity was enriched over 130,000-fold from a purified *L. tarentolae* mitochondrial fraction by a series of affinity, perfusion ion exchange, size-fractionation, and hydrophobic interaction chromatography columns. The isolation procedure is shown in Table 1 and Figure 1A. The final preparation had a single 135 kDa band and 2 minor bands of 65 and 75 kDa that varied in relative intensity in different isolations and did not react with anti-TUTase antiserum. It is of course possible that the presence of inhibitors would influence the calculated fold purification in the early fractionation stages, but this is unlikely since the activity was linear at all stages of purification.

Cloning, Sequencing, and Expression of TUTase Genes from *L. tarentolae* and *T. brucei*

Tryptic peptides from the 135 kDa protein were microsequenced and used to construct degenerate primers for PCR. The genes were cloned and sequenced from both *L. tarentolae* and *T. brucei*. The genes are single copy and the *L. tarentolae* gene was located on the ~600 kb chromosome 9 (data not shown).

His-tagged proteins were expressed in *E. coli* and used to generate polyclonal antisera. A 135 kDa band reacting with the *L. tarentolae* antiserum was detected at each stage of the biochemical isolation of the enzyme (Figure 1A, right panel).

The Recombinant *L. tarentolae* TUTase Is an Oligomer

Native gel analysis of the recombinant *L. tarentolae* TUTase (Figure 1B) showed a minor band of presumably unfolded protein migrating slightly above monomer molecular weight and a major band migrating at around 500 kDa. Biochemically purified TUTase (Figure 5B, lane1) migrated in a native gel as a single band of approximately 500 kDa. The recombinant and native (purification step IV) *L. tarentolae* TUTase eluted in Superose 6 gel filtration at approximately 500 kDa (not shown). The recombinant protein was treated with three crosslinking agents and subjected to SDS gel electrophoresis and Western blotting (Figure 1C). Three crosslinks of varied

[5]Correspondence: simpson@kdna.ucla.edu
[6]These authors contributed equally to this work.

Table 1. Isolation *L. tarentolae* Mitochondrial 3′ TUTase

	TUTase units (nmol UTP/min)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Enrichment	
Total cell extract	N/D	N/D	0.05	N/D	1	
Mito extract	420	2,160	0.19	100	3.8	
S100	390	540	0.7	93	14	I
Poly U	311	5.2	60	74	1,200	II
Poros Q	190	0.4	475	45	9,500	III
Superose 12	120	0.1	1200	29	24,000	IV
Phenyl Sepharose	40	0.006	6,670	10	134,000	V
Recombinant protein			8,200			

efficiency were observed. The size of the first crosslink corresponds to a dimer, and the larger two crosslinks most likely represent a trimer and tetramer. The gel filtration, native gel analysis, and crosslinking results are consistent with a tetrameric quaternary structure, but this must be confirmed by structural analysis. We will therefore refer to the conformation of the recombinant TUTase as "oligomeric."

TUTase Is a Member of the DNA Polymerase β Superfamily of Nucleotidyltransferases

Three functional domains were identified by PROSITE database searching (Figure 1D). The mononucleotide binding domain has the hG[GS] × (9-13) Dh[DE]h motif characteristic of the entire pol β superfamily (Aravind and Koonin, 1999). A "poly(A) polymerase core domain" and a "poly(A) polymerase-associated domain" were also identified. However, there are insertions of more than 150 amino acids between the second and third metal binding aspartates in TUTase from the two species, which are not found in poly(A) polymerase.

U-Specificity of TUTase

Recombinant proteins from both species showed a robust 3′ addition of Us to substrate RNAs, with little incorporation of ATP, a limited incorporation of CTP, and a limited incorporation of GTP that terminates predominantly at 14 G's, as shown in Figure 2A. This limita-

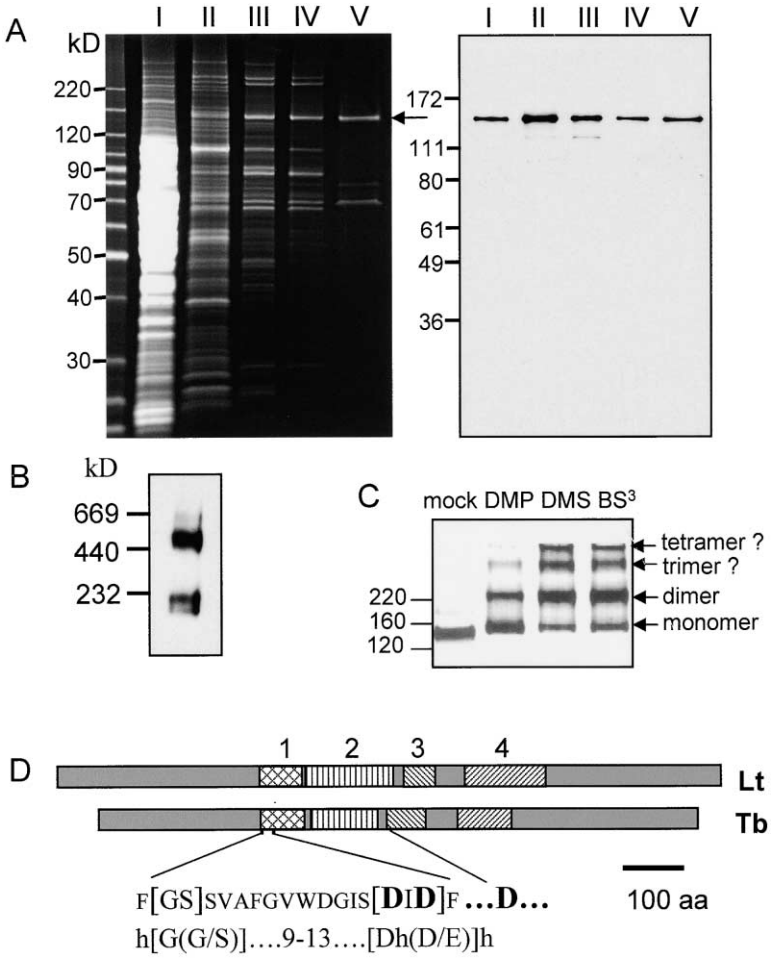

Figure 1. Purification of 3′ TUTase from *L. tarentolae* Mitochondria

(A) SDS gel analysis of fractionation steps. Approximately equal number of units of enzyme activity were loaded per lane. Left panel: Sypro ruby-stained gel. Right panel: Western blot with anti-TUTase antiserum. The numbers refer to the isolation step in Table 1.

(B) Western blot of recombinant TUTase separated on a native 8%–16% gel, visualized with PentaHis monoclonal antibody (Qiagen).

(C) Chemical crosslinking of recombinant TUTase with homobifunctional imidoesters with different chain lengths (10 Å for DMP, 11 Å for DMS, 11.4 Å for BS^3). Reaction products were separated on a 8%–16% SDS gel and detected with anti-TUTase antiserum.

(D) Diagram of the *L. tarentolae* and *T. brucei* TUTases. The nucleotidyltransferase superfamily core motif (Holm and Sander, 1995) is shown below with the metal binding aspartates in boldface. Motifs: (1) Mononucleotide binding. (2) Amino acid insertions between the second and third aspartates specific to the TUTase sequences. (3) Poly(A) polymerase core. (4) Poly(A) polymerase-associated.

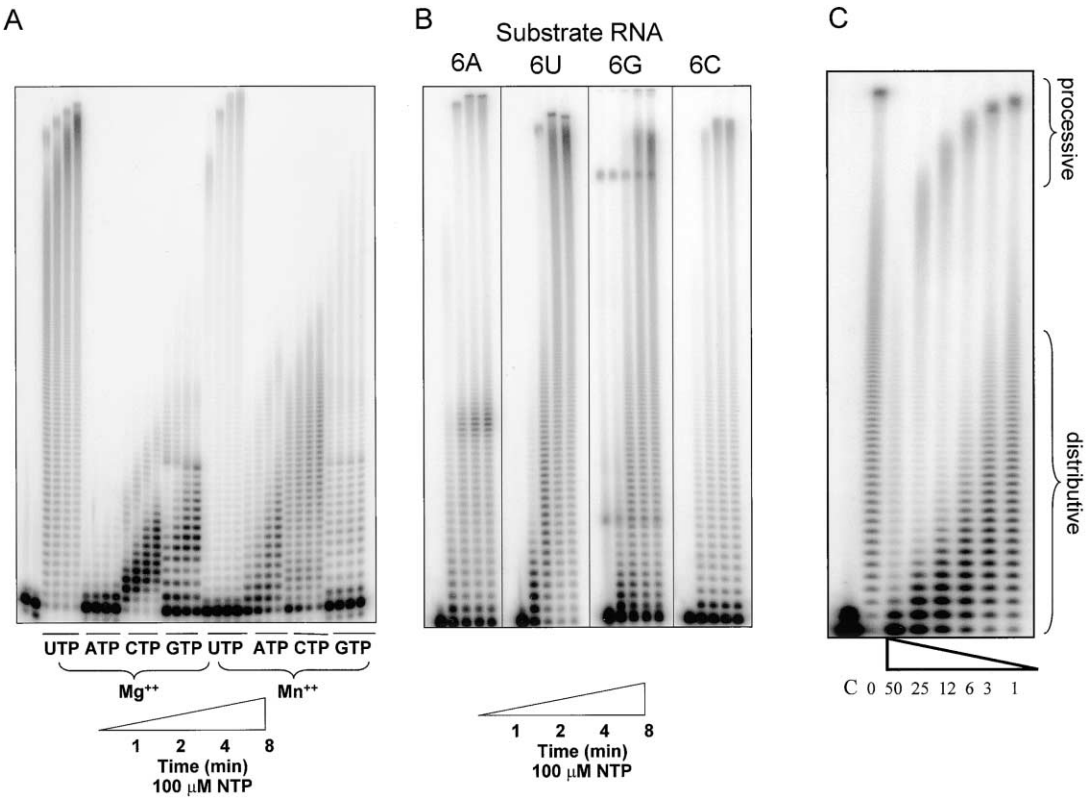

Figure 2. Enzymatic Properties of the *L. tarentolae* Recombinant TUTase

(A) 5′-labeled [G]12-U synthetic RNA (0.1 μM), NTPs (100 μM), and TUTase (0.2 U/ml) were incubated at 27°C and aliquots were removed at indicated times for gel analysis. The "Mg^{++}" reactions contained 10 mM Mg^{2+} and the "Mn^{2+}" reactions contained 2 mM Mn^{2+}.

(B) Effect of 3′-terminal nucleotides on TUTase activity. Substrates were synthetic RNAs ending as indicated. Reactions were run as in (A) for indicated times.

(C) Primer challenge assay: One pmol of 5′-labeled [G]12-U RNA and 5 mU of TUTase were preincubated in 20 μl reaction for 10 min before addition of UTP to 100 μM plus a 1- to 50-fold excess of the same unlabeled primer and a further incubation for 10 min. The products interpreted as processive and distributive transferase activity are indicated.

tion of G addition to 14 nucleotides has also been reported for poly(A) polymerase (Martin and Keller, 1998). Substitution of Mg^{2+} with Mn^{2+} affected the nucleotide specificity of the recombinant enzyme, giving rise to a substantial incorporation of both A and C in addition to U.

The minimal length of the RNA substrate was at least 10 nucleotides (not shown). However, at UTP concentrations above 10 μM, a low level of UTP self-polymerization was observed in the absence of substrate RNA (not shown). The *L. tarentolae* enzyme added Us to RNAs terminating in 6As, 6Cs, or 6Gs, but showed a preference for RNAs terminating in 6 Us (Figure 2B).

TUTase Exhibits Both Distributive and Processive Transferase Activities

Labeled RNA primer was preincubated with TUTase and then challenged with an excess of unlabeled primer plus UTP (Greider, 1991). Figure 2C shows the presence of both a minor processive transferase activity in which the formation of a high molecular weight product is not greatly affected by the unlabeled primer, and a major distributive transferase activity in which the addition of Us is inhibited by competition with unlabeled primer upon addition of each subsequent nucleoside. Substitution of Mg^{2+} by Mn^{2+} selectively inhibited the distributive activity of the enzyme (Mn^{2+} panel in Figure 2A). The absence of a continuous ladder between the "distributive" and the "processive" patterns also indicates that there are two types of activities. The shortening of the processive product by the excess primer probably indicates recycling of the enzyme after a number of processive catalytic acts.

TUTase Activity Is Modulated by the Nature of the RNA Substrate

The activity of the recombinant *L. tarentolae* TUTase on model editing substrates is shown in Figure 3. The substrates consisted of two short synthetic RNAs bridged by an RNA (brRNA) that was complementary both to the 3′ cleavage fragment (forming the "anchor duplex") and the 5′ cleavage fragment (Kapushoc and Simpson, 1999), and had a single strand gap with A or G nucleotides. Use of the 5′-end-labeled 5′ fragment alone as substrate led to the addition of a ladder of Us, the length of which was determined solely by the enzyme and UTP concentration. Annealing of the 5′ fragment and the brRNA somewhat decreased the number of Us added, but annealing the 5′ fragment, the 3′ fragment,

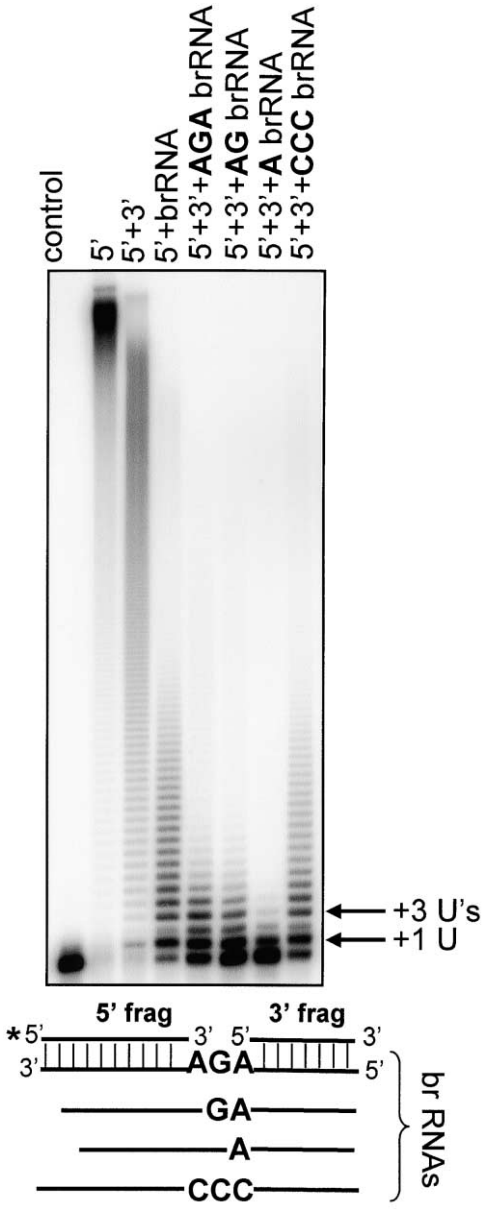

Figure 3. Model RNA Substrates with the Indicated Guiding Nucleotides in the brRNA Were Used in TUTase Reactions

* = 5′ end label. The "5′ + 3′" lane contained a 3-fold excess of unlabeled 3′ fragment. The Control lane contained no enzyme. The locations of the oligonucleotides with +1 and +3 Us are indicated.

and the brRNA had an inhibitory effect on the number of Us added. The size of the single-stranded gap also affected the number of Us added. There was a predominant +1 band with all substrates, especially with the A brRNA, but the AGA brRNA yielded in addition a predominant +3 U band, and the GA brRNA a +2 band and +3 in equal amounts. This inhibitory effect required guiding nucleotides that could base pair with the added Us as shown by the ladder produced with the non-base-pairing CCC brRNA. The +1 U activity was also observed with single-stranded RNA primers at low enzyme concentrations (<0.01 nM, not shown).

TUTase Is Localized in the Mitochondrion and TUTase Activity Can Be Completely Immunodepleted by Anti-TUTase Antiserum

A mitochondrial localization of TUTase (Bakalara et al., 1989) was experimentally confirmed by Western analysis of subcellular fractions. As shown in Figure 4A, TUTase was highly enriched in the gradient-purified mitochondrial fraction in lane 4, as was the mitochondrial matrix protein, glutamate dehydrogenase. Neither TUTase nor glutamate dehydrogenase were found in the cytosol fraction in lane 2. Tubulin, a component of the plasma membrane in *Leishmania*, was found in the crude mitochondrial membrane fraction in lane 3, but was not detected in the purified mitochondrial fraction.

Protein G Sepharose beads with bound affinity-purified mouse polyclonal antiserum against the *L. tarentolae* recombinant TUTase were used to immunodeplete mitochondrial extract. As shown in Figure 4B, this resulted in almost complete depletion of both TUTase activity and TUTase protein from the supernatant solution. Short synthetic RNAs were used as substrates in this assay, but no indication of any other TUTase activity was observed using a bridged RNA substrate (not shown). These data suggest that the identified protein is the only one responsible for the 3′ TUTase activity in *L. tarentolae* mitochondria.

The recombinant TUTase migrated faster in the SDS gel than the native enzyme (Figure 4B, lane 6), suggesting the presence of some type of modification that is lacking in the recombinant protein.

TUTase Binds Guide RNA and Interacts with RNA Ligase

A specific association of TUTase and the ATP-labeled p45 and p50 RNA ligase proteins (the homologs in *T. brucei* are termed p50 and p57 by Sabatini and Hajduk, TbMP48 and TbMP53 by Schnaufer et al., and Bands V and IV by Rusché et al.) (Sabatini and Hajduk, 1995; Rusché et al., 2001; Schnaufer et al., 2001; McManus et al., 2001; Peris et al., 1997) was shown by co-IP with anti-TUTase antiserum even in the presence of 1% Triton X-100 in 150 mM NaCl (Figure 4C, left panel). A control Western showed the IP of TUTase and the lack of co-IP of glutamate dehydrogenase (right panel), an abundant mitochondrial protein with RNA binding properties, but not involved in RNA editing (Bringaud et al., 1997; Estévez et al., 1999). However, virtually no depletion of p45 and p50 from the mitochondrial extract was observed even when all detectable TUTase was immunoprecipitated (data not shown), suggesting that the TUTase-ligase interaction involves only a portion of the ligase complexes.

Guide RNA, which can be specifically 5′-labeled with GTP and guanylyltransferase (Blum and Simpson, 1990), was also detected in the TUTase IP (Figure 4D, TUT lane). As a control for nonspecific RNA binding, the abundant mitochondrial $tRNA^{Ile}$ (Ex lane) was not detected in the TUTase IP (TUT lane). A direct interaction of synthetic labeled gRNA and TUTase in the mitochondrial extract was also shown by UV crosslinking followed by TUTase IP and RNase treatment (Figure 4E). A crosslink was observed with TUTase, which did not depend on the presence of a 3′ oligo(U) tail in the labeled substrate

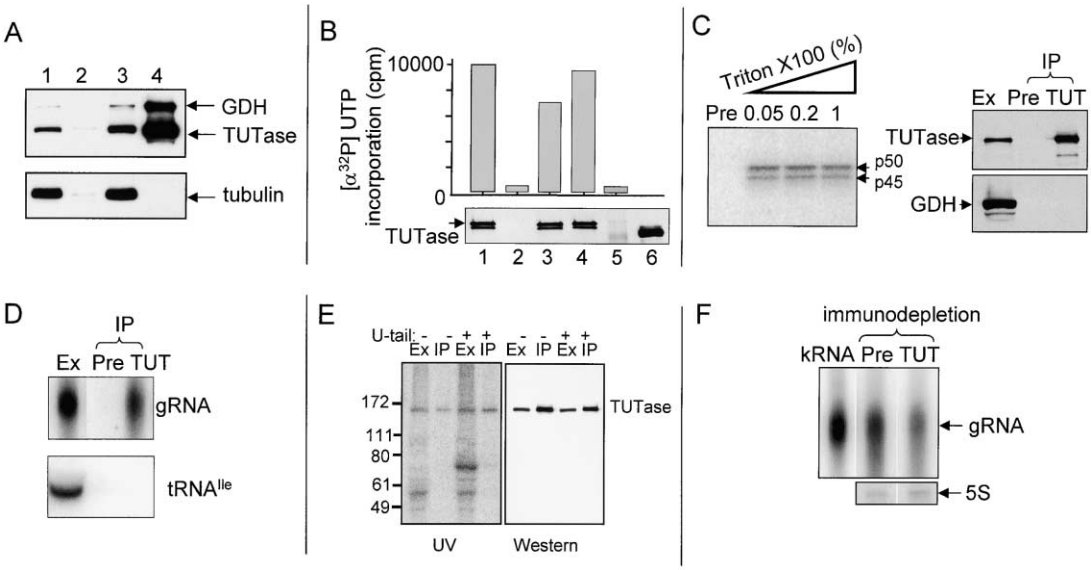

Figure 4. Analysis of Mitochondrial Extract Using Anti-TUTase Antiserum

(A) Mitochondrial localization of TUTase. Lane 1, total cell extract; lane 2, cytosol; lane 3, crude mitochondrial fraction; lane 4, density gradient purified mitochondria. Western blot of 10 μg of protein from each cell fraction using anti-TUTase, anti-GDH (Bringaud et al., 1997), and anti-tubulin antisera.

(B) Clarified extract was incubated with Protein G beads coated with affinity-purified mouse anti-TUTase antiserum. Upper panel: TUTase activity in the supernatant and bound to the beads. Lower panel: Western blot with anti-TUTase antiserum. Lane 1, mt extract; lane 2, mt extract after immunodepletion of TUTase activity; lane 3, TUTase activity bound to beads; lane 4, mt extract after immunodepletion with preimmune serum; lane 5, TUTase activity of beads with preimmune serum; lane 6, recombinant TUTase.

(C) Co-IP of p45 and p50 ligases with TUTase. Beads from lane 3 in (B) were incubated with [α-^{32}P]ATP to label p45 and p50. Bound proteins were separated on a 10%–20% SDS gel and transferred to nitrocellulose. The filters were exposed to a PhosphoImager screen (left panel) and then subjected to Western blotting with rabbit anti-TUTase antibody (TUTase) and anti-glutamate dehydrogenase antibody (GDH) (right panel). Ex, extract; Pre, preimmune serum; TUT, anti-TUTase antiserum; IP, immunoprecipitation.

(D) Co-immunoprecipitation of gRNAs with TUTase. RNA was isolated from the mitochondrial extract (Ex), the preimmune Co-IP material (Pre), and the anti-TUTase Co-IP material (TUT). gRNAs were specifically labeled with [α-^{32}P]GTP and guanylyltransferase. Upper panel: Autoradiogram of labeled gRNA from each fraction. Lower panel: Northern blot with probe for mitochondrial tRNA^{Ile}.

(E) UV-crosslinking of gRNA and TUTase. Mt extract was incubated with labeled in vitro transcribed gRNA (with or without a 3′ oligo(U) tail) in the presence of 0.1 mg/ml of nonspecific RNA (see Experimental Procedures), and irradiated with 254 nm UV. IP was performed with anti-TUTase antibody. Both extract and IP material were treated with RNase A and separated on an SDS gel and the TUTase band was detected by Western analysis.

(F) Immunodepletion of gRNA with anti-TUTase antiserum. S-100 mitochondrial extract was extracted three times with IgG of preimmune serum (Pre lane) or anti-TUTase antibody (TUTase lane). A control Western showed complete removal of the TUTase by the anti-TUTase extraction (not shown). RNA was isolated from the supernatant and labeled with [α-^{32}P]GTP and guanylyltransferase and subjected to acrylamide-urea gel electrophoresis. The bands were quantitated by PhosphoImager analysis. The "5 S" band is a GTP-labeled RNA in the same gel used as a loading control. The kRNA lane is total gRNA from untreated extract labeled with [α-^{32}P]GTP.

RNA. An unrelated synthetic RNA of the same length and lacking any stable predictable structure (see Experimental Procedures for sequence) produced a number of crosslinks with the mitochondrial extract, but no label transfer to TUTase protein was detected in the immunoprecipitated material (not shown).

The immunodepletion of gRNA from the extract by anti-TUTase antiserum was measured as shown in Figure 4F. Approximately 40% of the gRNA was depleted under conditions in which all detectable TUTase was depleted.

Separation of Two Classes of TUTase by Anion Exchange Chromatography

Two peaks of TUTase activity were observed in the Poros Q step of the enzyme isolation (Figure 5A). The major TUT I peak, which was used for further purification of the enzyme, sedimented in a glycerol gradient at approximately 10S (not shown), migrated in a native gel at around 500 kDa (Figure 5B, lane 1), and is likely to represent the oligomeric protein by itself. The minor TUT II peak sedimented in a glycerol gradient at approximately 20S (Figure 5C) and migrated in a native gel as a major band of approximately 700 kDa (Figure 5B, lane 2).

It was shown previously that the adenylated p50 and p45 RNA ligase proteins in the 20S region are components of a complex of ~1200 kDa (Rusche et al., 1997) and a minor complex of ~500 kDa (Peris et al., 1997), the relative amounts of which vary between preparations. An SDS gel of each column fraction of the TUT II peak, showing the elution of TUTase two fractions prior to the ATP-labeled p45 + p50, is presented in Figure 5A (inserted panels). A direct comparison of native gel profiles of TUTase and the ATP-labeled high molecular weight complex in the pooled TUT II peak confirmed that these represent distinct entities (Figure 5B, compare lanes 2 and 4). A glycerol gradient fractionation of the pooled TUT II peak also showed a clear separation

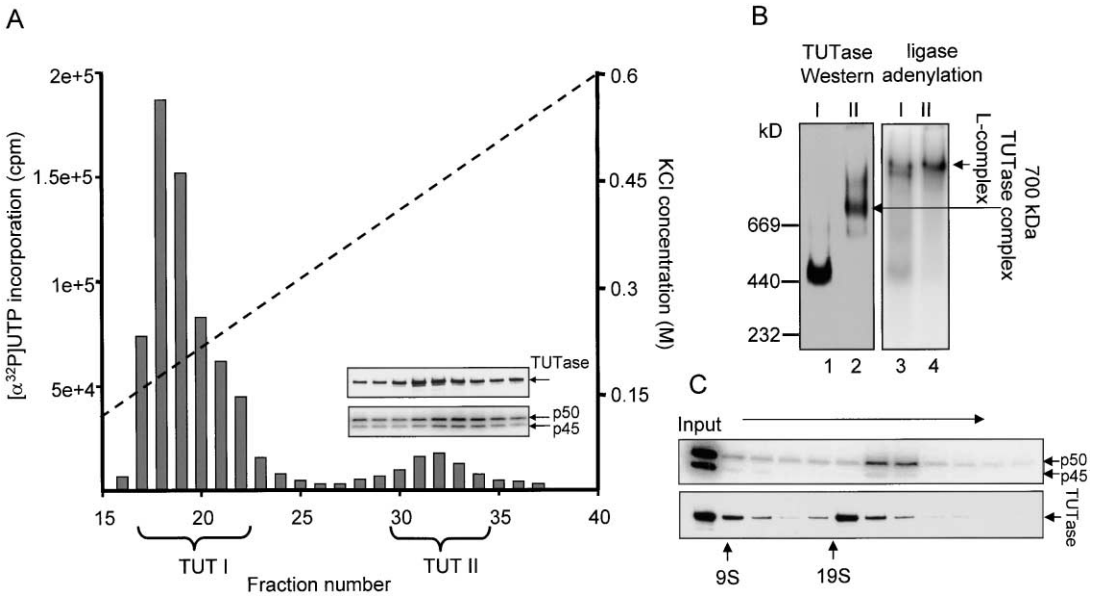

Figure 5. Two Forms of TUTase

(A) Two peaks of TUTase activity separated by anion exchange chromatography (step III in Table 1). The TUT I peak was used for the isolation of TUTase. The presence of TUTase and the p50 and p45 RNA ligases in the TUT II peak fractions are shown by Western analysis and by adenylation of p50 and p45 in each fraction.

(B) Native gel analysis of TUT I and TUT II fractions. I, TUT I peak. II, TUT II peak. Fractions indicated by brackets were pooled, subjected to adenylation to label p45 and p50, and separated on 8%–16% native gel. Left panel: Western using anti-TUTase antiserum. Right panel: Autoradiograph of ATP-labeled L-complex.

(C) Glycerol gradient sedimentation of TUT II. Fractions 30–34 from (A) were concentrated and centrifuged on 10%–30% glycerol gradient in an SW41 Beckman rotor for 20 hr at 35,000 RPM. Each fraction was ATP-labeled to visualize the p45 and p50 ligases, and the gel was blotted, exposed to a PhosphoImager screen, and treated with anti-TUTase antiserum.

of the TUT II TUTase and the ATP-labeled band (Figure 5C). Since the ATP-labeled band contains p45 and p50 and comigrated in a native gel with the ATP-labeled complex from glycerol gradients (not shown), we interpret this as the 1200 kDa complex (Peris et al., 1997), which has survived ion exchange chromatography. These fractionations show that both TUTase and RNA ligase form stable but distinct higher-order complexes that can withstand the conditions of ion-exchange chromatography (high salt and the absence of Mg^{2+}). However, the coimmunoprecipitation of RNA ligase from mitochondrial extract with anti-TUTase antibody (Figure 4C) clearly demonstrates that TUTase interacts with a minor fraction of the total RNA ligase.

Two Classes of TUTase Are Also Separated by Glycerol Gradient Sedimentation and the Higher Molecular Weight Class Is Sensitive to RNase Digestion

Glycerol gradient sedimentation of mitochondrial extract and Western analysis of each fraction using anti-TUTase antiserum is shown in Figure 6A. The SDS gel profile (Figure 6A, panel 2) showed the distribution profile described previously for TUTase activity (Peris et al., 1997). Native gel analysis of each fraction (Figure 6A, panel 1) showed that the 10S peak (labeled I) contains the TUTase oligomer as a major component but is more heterogeneous than the TUT I TUTase, probably as a result of the removal of bound RNA and other proteins by the ion exchange conditions. The TUTase sedimenting around 20S migrated in a native gel as a high molecular weight smear of around 700 kDa (Figure 6A, II), consistent with this component being equivalent to the 700 kDa TUTase complex detected in the TUT II peak after ion exchange chromatography (Figure 5B). In addition, a minor amount of TUTase complexes with S values greater than that of the TUT II complex were also detected (Figure 6A).

Pretreatment of the extract with RNase prior to sedimentation resulted in a loss of the higher molecular weight TUTase material (Figure 6B, II). There was less effect of RNase treatment on the lower molecular weight TUTase material. This evidence suggests the presence of an RNA component in the 700 kDa TUT II TUTase complex that is required for stability. An obvious candidate is the gRNA shown above by coimmunoprecipitation and UV crosslinking to be associated with TUTase.

Effect of Inhibition of TUTase Expression in *T. brucei* Procyclic Cells

Since a workable conditional gene expression system is not available in *Leishmania*, the effect of RNAi of TUTase expression was examined in procyclic *T. brucei* (Ngo et al., 1998) using an integrated expression vector containing head-to-head fragments of TUTase under control of a tetracycline-inducible PARP promoter (LaCount et al., 2000). Induction of RNAi by addition of tetracycline led to a severe growth defect after 5–6 days (Figure 7A).

The level of TUTase protein decreased rapidly after induction of RNAi with tetracycline whereas neither the

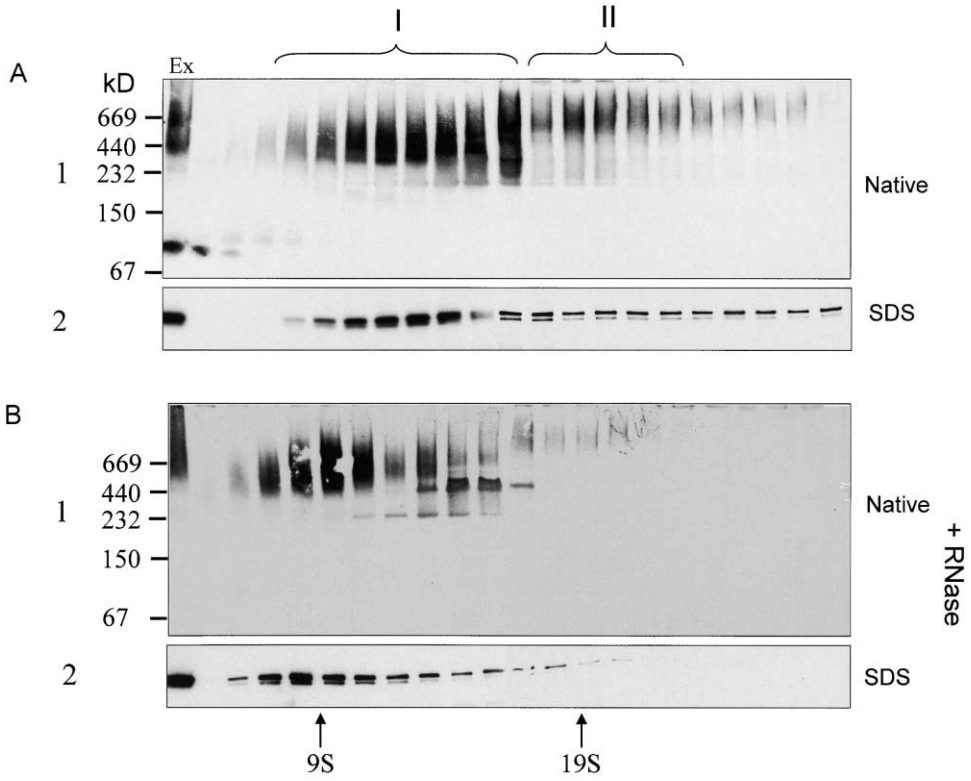

Figure 6. Glycerol Gradient Sedimentation of TUTase in Mitochondrial Extract

(A) Untreated mitochondrial extract was sedimented in a 10%–30% glycerol gradient and fractions were electrophoresed in a native 8%–16% acrylamide gel (panel 1) or an SDS acrylamide gel (panel 2). The gels were blotted and probed with anti-TUTase antiserum. The regions labeled I and II correspond to the TUT I and TUT II TUTase peaks, respectively.

(B) The mitochondrial extract was digested with RNase A prior to sedimentation.

29-13 parental cells (Wirtz et al., 1999) treated with tetracycline nor the cells not treated with tetracycline showed any changes in TUTase abundance (Figure 7B). The effect on editing was analyzed for 6 out of the 12 edited mRNAs using labeled primers that hybridized just downstream of the editing domains and were extended either to the 5′ end of the mRNA (Cyb and Murf2 mRNAs) or to a specific residue where termination was achieved with incorporation of a dideoxynucleotide (CO2, ND8, and CO3 mRNAs). The time course of the effect on editing of the ND7 mRNA is shown in Figure 7C. There is a decrease in the ratio of the edited to preedited bands with time of induction of RNAi, reaching approximately 75%. An absence of a general effect on the stability or transcription of mitochondrial RNA was shown by the primer extension of the never edited CO1 mRNA and the cytosolic calmodulin mRNA (Figure 7C, lower panels). Based on this result, an internal control was performed for the ND7 mRNA by incorporating a primer for the CO1 mRNA in the reaction (Figure 7C, upper panel), thereby allowing a normalization of loading. Little change was observed in the overall levels of edited + preedited mRNAs during the RNAi induction, suggesting no effect of RNAi on stability of edited or preedited RNA.

The primer extension results for CO3, Cyb, Murf2, and ND8 are shown in Figure 7D. In each case, there was approximately a 90% decrease in the percentage of edited transcripts by 142 hr of RNAi induction. The effect on the editing of the CO2 mRNA was less pronounced but significant. As shown in Figure 7E, there was a maximum 30% decrease in the percentage of edited transcripts.

Discussion

Three motifs characteristic of the poly(A) polymerase N-terminal catalytic core and central regions (Martin and Keller, 1996; Martin et al., 1999, 2000; Bard et al., 2000) are conserved in the *Leishmania* and *Trypanosoma* TUTase proteins. We have preliminary evidence that mutation of the conserved aspartates in positions 342, 344, and 548 completely inactivates the enzyme, while mutation of neighboring aspartates has no effect, confirming the functional importance of these residues and the general similarity of 3′-uridine addition to other types of nucleotidyltransferase activities. However, an insertion of more than 200 amino acids between the second and third aspartate residues of the metal binding triad appears to be unique to TUTase. The oligomeric structure of the *L. tarentolae* TUTase is also not typical of other members of the superfamily, which are generally monomeric.

The TUTase enzymes from both species show a high degree of specificity for UTP with a K_m of approximately 50 μM (not shown). This nucleotide specificity was decreased by substitution of the Mg^{2+} metal cofactor with Mn^{2+}, as was previously observed for poly(A) polymerase (Chen and Moore, 1992). The enzyme in the pres-

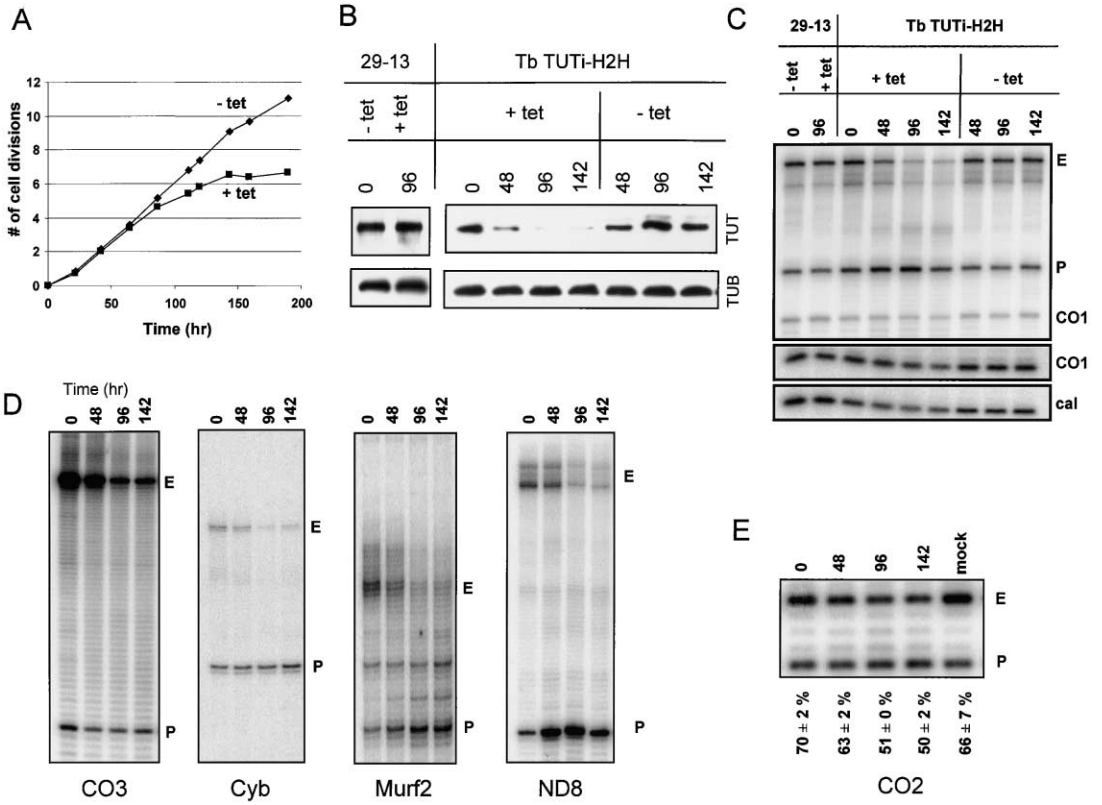

Figure 7. RNAi of TUTase Expression Affects RNA Editing In Vivo

RNAi was induced in procyclic TbTUTi-H2H cells by the addition of 1 μg/ml tetracycline. The parental 29-13 cell line was treated identically for 96 hr.

(A) Cumulative growth curve with and without tetracycline. Cell growth is expressed as number of cell divisions.

(B) Western analysis of induced and mock-treated cells using anti-TUTase antiserum. Each lane had 15 μg of total cell protein. Tubulin was used as a loading control.

(C–E) Primer extension analyses of mitochondrial mRNAs (20 μg of total RNA per lane).

(C) Time course of the analysis of ND7 mRNA for the induced and mock-treated TbTUTi-H2H and 29-13 cell lines. CO1 mRNA was analyzed in the same primer extension reaction as an internal control for loading and assay performance. Lower panels: Primer extension analysis of the never edited CO1 and the cytosolic calmodulin mRNA, showing no change of steady-state levels of mitochondrial transcripts.

(D) Analysis of changes in editing patterns of CO3, Cyb, Murf2, and ND8 mRNAs during RNAi induction. E, edited; P, preedited.

(E) Change of editing of CO2 mRNA. The extension products were quantified and the percentage of editing was calculated (n = 4).

ence of Mg^{2+} functions mainly in a distributive mode, but at high UTP concentrations can processively add several hundred Us to the 3′ end of the RNA substrate. The nature of the terminal nucleotides of the RNA primer have little effect on the TUTase reaction except for a preference for a 3′ string of Us. This property is consistent with a role of TUTase in addition of Us at an editing site since the 5′ adjacent nucleotide is G or A with an occasional C.

The number of Us added to a bridged RNA substrate by the recombinant *L. tarentolae* TUTase was limited by the size of the gap between the 5′ and 3′ cleavage fragments, provided that the guiding nucleotides could base pair with the added Us. Furthermore, the mitochondrial RNA ligase activity, either partially purified (Blanc et al., 1999) or in total mitochondrial extract (Igo et al., 2000), has been shown to have a preference for ligating a bridged nicked substrate rather than one with a gap or a bulge. We propose that the substrate specificity of TUTase combined with that of the RNA ligase may represent the major factors determining the precise number of Us added at an editing site. Of course, this hypothesis does not preclude the possibility that additional protein factors are involved with determining the extent of processivity of the TUTase at an editing site, as in the case of poly(A) polymerase (Sachs and Wahle, 1993), or the possibility that the U-specific 3′-exonuclease (Aphasizhev and Simpson, 2001) serves to trim a 3′ oligo(U) overhang (Alfonzo et al., 1997). A similar hypothesis was proposed by Igo et al. (2000) based on results obtained using a crude mitochondrial extract from *T. brucei* and a precleaved in vitro editing assay.

Two peaks of TUTase activity sedimenting at ~10S and ~20S in glycerol gradients of mitochondrial extracts from both *L. tarentolae* and *T. brucei* were reported previously (Peris et al., 1997). In this paper, we characterized two stable forms of the mitochondrial TUTase (TUT I and TUT II) that can survive anion exchange chromatography and/or glycerol gradient sedimentation. TUT I migrates in a native gel at around 500 kDa and represents the TUTase oligomer. The TUTase in the TUT I and the 10S glycerol gradient fraction could represent

a precursor for the 700 kDa TUTase complex or a breakdown product, or it could play a role in the 3′ addition of Us to the gRNAs (Blum and Simpson, 1990), mRNAs (Campbell et al., 1989), and rRNAs (Adler et al., 1991), which represent other roles for TUTase in these cells in addition to the insertion of Us at editing sites.

The TUT II complex migrates in a native gel at ~700 kDa, which suggests the presence of additional components. We have presented immunodepletion and cross-linking evidence for the binding of approximately 40% of total gRNA by TUTase or a TUTase-containing complex. We also showed the presence of an RNA component required for stability of the TUT II complex by the loss of this complex after treatment of the extract with RNase. This is consistent with previous micrococcal nuclease digestion evidence for a requirement of an RNA component for a gRNA-independent in vitro U-insertion activity in an *L. tarentolae* mitochondrial extract (Frech et al., 1995).

Chromatographic evidence for the presence of two TUTase activities in *T. brucei* mitochondrial extract has been previously presented, and it was suggested that there might be two TUTase enzymes with different biological functions (McManus et al., 2000). Our data, however, suggest that there is a single mitochondrial TUTase in both trypanosomatid species, which is present in at least two states.

The decrease in the relative abundances of several edited mRNAs, which correlated with inhibition of TUTase expression by RNAi in procyclic *T. brucei*, provide evidence for a possible role of this enzyme in editing. Since gene rescue with expression of a mutant of TUTase that is functional but not degraded by RNAi is technically difficult, it is of course a theoretical possibility that other mRNAs with similar motifs in the region of or upstream of the 560 bp double-stranded RNA are also degraded, and that the decrease in TUTase expression is not the primary cause for the decrease in editing and loss of viability (Lipardi et al., 2001; Sijen et al., 2001). This is unlikely since Southern blot analysis showed a single copy gene, and Blast analysis of the database with this sequence did not find any significant similarities, other than TUTase homologs from related trypanosomatids (not shown). Additional evidence for TUTase being a vital gene was the fact that we could only knock out both alleles in the presence of an ectopic copy. In addition, we have preliminary evidence for the correlation of immunodepletion of a TUTase-containing complex with a loss of in vitro editing activity, which would limit any possible additional target of TUTase RNAi to a component of this complex. Only a few components have yet been identified, but the known ones, TbMP52 and TbMP48 RNA ligases (Panigrahi et al., 2001a) and gBP21 and gBP25 RNA binding proteins (Koller et al., 1997; Blom et al., 2001) have no similarity with TUTase, nor do several possible components, TbMP18, TbMP42, TbMP63, TbMP81 (Panigrahi et al., 2001b), TbmHel61p (Missel et al., 1997), TbREAP-1 (Madison-Antenucci et al., 1998), and TbRGG1p (Vanhamme et al., 1998). This suggests that the observed effect on editing in the RNAi experiments was most likely due solely to the degradation of TUTase mRNA.

The loss of viability in the TUTase RNAi cells appears to be a consequence of a decrease in translation of edited mRNAs. We have preliminary evidence that this leads to an effect on the formation or stability of respiratory complexes and a subsequent effect on respiratory metabolism.

It should be noted that the RNAi data cannot distinguish between a direct effect on U-insertions at the editing sites or an indirect effect involving the inhibition of U-addition to the 3′ end of the gRNAs and mRNAs and a subsequent effect on the editing process. However, the fact that inhibition of editing was observed for the CO2 mRNA, in which the gRNA is the 3′ end of the mRNA and functions in *cis* in the absence of an oligo(U) tail, suggests that the inhibition of TUTase expression directly affects insertion of Us at the editing sites. Since we have shown that there is a single TUTase enzyme in the mitochondrion, this must also be responsible for the 3′ addition of nontemplated Us to gRNAs, rRNAs, and the addition of Us to the 3′ poly(A) tail of mRNAs. The biological functions of these RNA modifications are not yet clear, but there is some evidence that the presence of Us in the poly(A) tail of mRNAs invokes a degradation pathway (Militello and Read, 2000). Our primer extension data, however, indicate no changes in the turnover of unedited, preedited, or edited mRNAs in the RNAi-induced cells.

The isolation of the mitochondrial TUTase involved in RNA editing should open the door to a detailed molecular analysis of this phenomenon and this work is in progress.

The fact that TUTase is a vital gene for procyclic *T. brucei* and that editing may also be required for viability of bloodstream *T. brucei* (Schnaufer et al., 2001) suggests that this enzyme may be an important drug target for selective chemotherapy of trypanosomatid-caused diseases such as African Sleeping Sickness, Leishmaniasis, and Chagas Disease. And finally, the presence of TUTase activity in a variety of eukaryotes not known to possess U-insertion/deletion RNA editing may indicate other functional roles for terminal U-addition to RNA molecules (Trippe et al., 1998).

Experimental Procedures

See the Cell web site for Supplementary material – detailed experimental procedures (http://www.cell.com/cgi/content/full/108/5/637/DC1).

Leishmania Culture and Mitochondria Isolation

Mitochondria were isolated by the hypotonic lysis Renografin density gradient method (Braly et al., 1974). *L. tarentolae* cells were grown in 15 liter batches in brain heart infusion medium to late log phase (~180 × 10^6 cells/ml) in a BioFlow IV Fermentor (New Brunswick). Approximately 10–12 g (wet weight) mitochondria were obtained from 15 liters cell culture. Approximately 50–100 g (wet weight) mitochondria were used for pilot fractionation experiments.

Trypanosome Culture and RNAi

To construct the pTUTi-H2H vector for inducible RNAi, two 560 bp PCR fragments containing nt 444–1024 of the *T. brucei* TUTase coding region were inserted in a head-to-head configuration with an internal stuffer fragment, under control of a tetracycline-regulatable PARP promoter (LaCount et al., 2000). The vector was transfected into procyclic *T. brucei* strain 29–13 (Wang et al., 2000), and resistant cell lines were selected by plating on agarose (Carruthers and Cross, 1992). Cells were cultured in SDM-79 medium and RNAi was induced with 1 μg/ml tetracycline. Cells were maintained in log phase growth by frequent dilution.

RNA Analysis

Total RNA was purified by the acid guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Primer extension was performed as described previously (Estévez et al., 1999). Cyb (3812) and Murf2 (3807) mRNAs were analyzed by run off extensions. CO1 (3808), CO2 (3809), CO3 (4080), ND7 (4282), ND8 (4130), and calmodulin (3813) mRNAs were analyzed by poisoned primer extension, using ddGTP. For normalization experiments, primers 3813, 3808, or 4282 were extended in the same reaction.

3807: 5′-CAACCTGACATTAAAAGAC-3′
3808: 5′-GTAATGAGTACGTTGTAAAACTG-3′
3809: 5′-ATTTCATTACACCTACCAGG-3′
3812: 5′-GTTCTAATACATAACAAATCAAAAACACG-3′
3813: 5′-GTTGATCGGCCATCGTAAATCAAGTGGATG-3′
4080: 5′-AACTTTTCCTACAAACTACC-3′
4130: 5′-GTCAAAATTTAATTTCACCGTG-3′
4282: 5′-CTTTTCTGTACCACGATGC-3′

Guide RNAs were specifically labeled with guanylyltransferase in the presence of [α-P^{32}]GTP. The 20 μl reaction contained 20 mM Tris-HCl (pH 7.8), 1.2 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 10 μCi of [α-P^{32}]GTP, 10–100 ng of mitochondrial RNA, and 5 U of enzyme. After 15 min incubation, the reaction was extracted with phenol, ethanol precipitated, and analyzed on a 10% denaturing gel.

Purification of 3′ TUTase from *L. tarentolae* Mitochondria

Enzymatic activity was measured as incorporation of an [α- ^{32}P]UTP (New England Nuclear) into synthetic [G]12-U RNA. Typically, 1 μl of protein fraction was added to a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM magnesium acetate, 1 mM DTT, 0.1 mM of [α- ^{32}P]UTP (1000–2000 cpm/pmol), 1 μM of RNA primer, and incubated at 27°C for 10 min. The reaction was stopped with 15 μl of 0.5 M sodium phosphate, 0.5% SDS, spotted on DE81 nitrocellulose filters, washed with 0.5 M sodium phosphate, and counted in a Beckman Scintillation Counter. The final concentration of 5′-labeled primers was kept at 0.1 μM and recombinant TUTase at 25–50 mU/ml. For bridged substrates, RNAs were annealed by heating in water to 90°C and slowly cooling to 20°C and the reaction was carried out for 30 min at 50–100 mU/ml. For the final isolation protocol, 40 g wet weight mitochondria (2.2 g protein) were extracted with 120 ml of 50 mM HEPES, (pH 7.2), 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, and 1 mM CHAPS (Roche) by sonication. The S-100 extract was subjected to successive chromatography on poly[U] Sepharose 4B (AP Biotech), Poros 20 HQ (Perceptive Biosystems), Superose 12-Superose 6 joint column (AP Biotech), and Phenyl Superose (AP Biotech). The 135 kDa Coomassie-stained band comigrating with TUTase activity was used for in-gel digestions and peptide microsequencing (Harvard Microsequencing Facility).

Cloning of TUTase Genes and Expression of Recombinant Proteins

Degenerate oligonucleotides based on the sequences of six peptides were used in all possible combinations for PCR amplification with *L. tarentolae* genomic DNA. The PCR fragment between two peptides (LVPVWDEVLK and AGFSFINLEPISHAAR) contained an open reading frame and was used to clone a 5.9 kb fragment which contained a 3360 nt ORF. The amino acid sequence was used to identify a genomic fragment from *T. brucei* encoding the entire gene.

The *L. tarentolae* TUTase gene was cloned into the pET29a vector (Novagen) and six histidine codons upstream of the stop codon were introduced by PCR. The plasmid was transformed into *E. coli* BL 21 Codon Plus RIL (Stratagene). Expression was induced with 1 mM IPTG and the temperature was lowered to 25°C for 3 hr. The recombinant protein was isolated by chromatography on Sepharose S (AP Biotech), a Talon metal affinity column (Clontech), and on Mono Q and Mono S. A final chromatography on Superose 6 yielded a protein more than 95% pure. The *T. brucei* TUTase was expressed using the pET43a(+) vector.

Western Blotting and Immunoprecipitation

Rabbit and mouse polyclonal antibody were raised against purified recombinant proteins (Covance Research). Antibodies were affinity purified on immobilized antigen columns. The mouse antibody was bound to GammaBind Protein G Plus Sepharose (AP Biotech), and incubated with S100 mitochondrial extracts and glycerol gradient fractions. IPs were performed for 2 hr at 4°C. For adenylation, the washed beads were incubated with 50 μCi/ml [α-^{32}P]ATP for 30 min at 27°C in 1× TUTase reaction buffer. RNA was isolated by SDS-phenol extraction and proteins extracted by SDS loading buffer. Western blotting was performed using the SuperSignal West Pico chemiluminescent system (Pierce). E7 anti-tubulin monoclonal antibody was from the Developmental Studies Hybridoma Bank of the University of Iowa.

Chemical Crosslinking

Protein-protein chemical crosslinking was performed as described (Pierce). Products were analyzed on 8%–16% SDS gel followed by Western blotting. DMP, DMS, and BS^3 were obtained from Pierce.

RNA Crosslinking

Uniformly labeled gND7-II gRNA (Simpson et al., 1998) with or without 13 Us at the 3′ end was synthesized by T7 transcription in the presence of [α-^{32}P]ATP. The labeled RNA was incubated with clarified mitochondrial extract in the presence of 0.2 mg/ml of fragmented denatured tRNA and UV irradiated for 5 min at 0.12 J/cm^2. IP was performed as described and the washed beads treated with RNase A (0.1 mg/ml, 20 min at 37°C) prior to SDS-gel electrophoresis. The gels were blotted and the filters exposed to a Phosphol-mager screen and reacted with anti-TUTase antibody. The following unstructured RNA was used as a specificity control: GGAGAGCCAG GAGAAAGAACACACGGAACAGUUUUCGAAAGCAACAAGCAACA GGAAAA.

Extract Preparation, Glycerol Gradient Sedimentation, and Native Gel Electrophoresis

Purified mitochondria (25 mg protein/ml) were lysed with 0.3% Triton X-100 in 10 mM Tris-HCl (pH 8.0), 125 mM sucrose, 10 mM $MgCl_2$, and 60 mM KCl. The clarified extract (300 μl) was centrifuged on a 10%–30% glycerol gradient in the SW41 rotor (Beckmann) for 20 hr at 35,000 rpm. The reaction was diluted 2-fold with water and electrophoresed on an 8%–16% native gel (Novex), or stopped with SDS loading buffer for 10%–20% denaturing gradient gels, and the gels blotted for Western analysis. Sedimentation values were calculated using aldolase (9S) and thyroglobulin (19S). It should be noted that we use the terms "20S"and "10S" to indicate the two major classes of TUTase and ligase reported by most laboratories and not to indicate precise S values.

RNA Substrates

RNA substrates were chemically synthesized (Oligos Etc., Inc, or Xeragon) and gel-purified.

[G]12-U: GCUAUGUCUGCUAACUUGUUUUUUUUUUUU
6-U: GCUAUGUCUGCUAACUUGUUUUUU
6-C: GCUAUGUCUGCUAACUUGCCCCCC
6-A: GCUAUGUCUGCUAACUUGAAAAAA
6-G: GCUAUGUCUGCUAACUUCGGGGGG
5′ fragment: CGACUACACGAUAAAUAUAAAAAG
3′ fragment: AACAUUAUGCUUCUUCGdC
AGA brRNA: AGAAGCAUAAUGUU*AGA*CUUUUUAUAUUUAUCG UGUAGUCdG
AG brRNA: AAGAAGCAUAAUGUU*AG*CUUUUUAUAUUUAUCGU GUAGUCdG
A brRNA: AAGAAGCAUAAUGUU*A*CUUUUUAUAUUUAUCGUGU AGUCdG
CCC brRNA: AAGAAGCAUAAUGUU*CCC*CUUUUUAUAUUUAUCG UGUAGUCdG

RNAs were 5′-phosphorylated with T4 polynucleotide kinase (BRL) and [$\gamma-^{32}$P]ATP. Complementary RNAs were annealed by heating and slow cooling.

Acknowledgments

This work was partially supported by a grant from the National Institutes of Health (AI09102) to L.S. We thank Kent Hill for the pLEW100-HX-GFP RNAi plasmid, and George Cross for the 29–13 strain of *T. brucei*. We thank all members of the Simpson laboratory for advice and assistance and Xuedong Kang for isolation of the

guanyltransferase expressed from the pET-His-D1/D12 plasmid, which was kindly provided by Stewart Shuman.

Received: April 25, 2001
Revised: January 16, 2002

References

Adler, B.K., Harris, M.E., Bertrand, K.I., and Hajduk, S.L. (1991). Modification of *Trypanosoma brucei* mitochondrial rRNA by post-transcriptional 3′ polyuridine tail formation. Mol. Cell. Biol. *11*, 5878–5884.

Alfonzo, J.D., Thiemann, O., and Simpson, L. (1997). The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. Nucleic Acids Res. *25*, 3751–3759.

Andrews, N., and Baltimore, D. (1986). Purification of a terminal uridylyltransferase that acts as host factor in the in vitro poliovirus replicase reaction. Proc. Natl. Acad. Sci. USA *83*, 221–225.

Aphasizhev, R., and Simpson, L. (2001). Isolation and characterization of a U-specific 3′–5′ exonuclease from mitochondria of *Leishmania tarentolae*. J. Biol. Chem. *276*, 21280–21284.

Aravind, L., and Koonin, E.V. (1999). DNA polymerase beta-like nucleotidyltransferase superfamily: identification of three new families, classification, and evolutionary history. Nucleic Acids Res. *27*, 1609–1618.

Bakalara, N., Simpson, A.M., and Simpson, L. (1989). The *Leishmania* kinetoplast-mitochondrion contains terminal uridylyltransferase and RNA ligase activities. J. Biol. Chem. *264*, 18679–18686.

Bard, J., Zhelkovsky, A.M., Helmling, S., Earnest, T.N., Moore, C.L., and Bohm, A. (2000). Structure of yeast poly(A) polymerase alone and in complex with 3′-dATP. Science *289*, 1346–1349.

Blanc, V., Alfonzo, J.D., Aphasizhev, R., and Simpson, L. (1999). The mitochondrial RNA ligase from *Leishmania tarentolae* can join RNA molecules bridged by a complementary RNA. J. Biol. Chem. *274*, 24289–24296.

Blom, D., Burg, J., Breek, C.K., Speijer, D., Muijsers, A.O., and Benne, R. (2001). Cloning and characterization of two guide RNA-binding proteins from mitochondria of *Crithidia fasciculata*: gBP27, a novel protein, and gBP29, the orthologue of *Trypanosoma brucei* gBP21. Nucleic Acids Res. *29*, 2950–2962.

Blum, B., and Simpson, L. (1990). Guide RNAs in kinetoplastid mitochondria have a nonencoded 3′ oligo-(U) tail involved in recognition of the preedited region. Cell *62*, 391–397.

Braly, P., Simpson, L., and Kretzer, F. (1974). Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. J. Protozool. *21*, 782–790.

Bringaud, F., Stripecke, R., Frech, G.C., Freedland, S., Turck, C., Byrne, E.M., and Simpson, L. (1997). Mitochondrial glutamate dehydrogenase from *Leishmania tarentolae* is a guide RNA-binding protein. Mol. Cell. Biol. *17*, 3915–3923.

Campbell, D.A., Spithill, T.W., Samaras, N., Simpson, A., and Simpson, L. (1989). Sequence of a cDNA for the ND1 gene from *Leishmania* major: potential uridine addition in the polyadenosine tail. Mol. Biochem. Parasitol. *36*, 197–200.

Carruthers, V.B., and Cross, G.A. (1992). High-efficiency clonal growth of bloodstream- and insect-form *Trypanosoma brucei* on agarose plates. Proc. Natl. Acad. Sci. USA *89*, 8818–8821.

Chen, J., and Moore, C. (1992). Separation of factors required for cleavage and polyadenylation of yeast pre-mRNA. Mol. Cell. Biol. *12*, 3470–3481.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Estévez, A.M., and Simpson, L. (1999). Uridine insertion/deletion RNA editing in trypanosome mitochondria - a review. Gene *240*, 247–260.

Estévez, A.M., Kierszenbaum, F., Wirtz, E., Bringaud, F., Grunstein, J., and Simpson, L. (1999). Knockout of the glutamate dehydrogenase gene in bloodstream *Trypanosoma brucei* in culture has no effect on editing of mitochondrial mRNAs. Mol. Biochem. Parasitol. *100*, 5–17.

Frech, G.C., Bakalara, N., Simpson, L., and Simpson, A.M. (1995). In vitro RNA editing-like activity in a mitochondrial extract from *Leishmania tarentolae*. EMBO J. *14*, 178–187.

Greider, C.W. (1991). Telomerase is processive. Mol. Cell. Biol. *11*, 4572–4580.

Holm, L., and Sander, C. (1995). DNA polymerase beta belongs to an ancient nucleotidyltransferase superfamily. Trends Biochem. Sci. *20*, 345–347.

Igo, R.P., Palazzo, S.S., Burgess, M.L., Panigrahi, A.K., and Stuart, K. (2000). Uridylate addition and RNA ligation contribute to the specificity of kinetoplastid insertion RNA editing. Mol. Cell. Biol. *20*, 8447–8457.

Kapushoc, S.T., and Simpson, L. (1999). In vitro uridine insertion RNA editing mediated by *cis*-acting guide RNAs. RNA *5*, 656–669.

Koller, J., Muller, U.F., Schmid, B., Missel, A., Kruft, V., Stuart, K., and Goringer, H.U. (1997). *Trypanosoma brucei* gBP21. An arginine-rich mitochondrial protein that binds to guide RNA with high affinity. J. Biol. Chem. *272*, 3749–3757.

LaCount, D.J., Bruse, S., Hill, K.L., and Donelson, J.E. (2000). Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. Mol. Biochem. Parasitol. *111*, 67–76.

Lipardi, C., Wei, Q., and Paterson, B.M. (2001). RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell *107*, 297–307.

Madison-Antenucci, S., Sabatini, R.S., Pollard, V.W., and Hajduk, S.L. (1998). Kinetoplastid RNA-editing-associated protein 1 (REAP-1): a novel editing complex protein with repetitive domains. EMBO J. *17*, 6368–6376.

Martin, G., and Keller, W. (1996). Mutational analysis of mammalian poly(A) polymerase identifies a region for primer binding and catalytic domain, homologous to the family X polymerases, and to other nucleotidyltransferases. EMBO J. *15*, 2593–2603.

Martin, G., and Keller, W. (1998). Tailing and 3′-end labeling of RNA with yeast poly(A) polymerase and various nucleotides. RNA *4*, 226–230.

Martin, G., Jeno, P., and Keller, W. (1999). Mapping of ATP binding regions in poly(A) polymerases by photoaffinity labeling and by mutational analysis identifies a domain conserved in many nucleotidyltransferases. Protein Sci. *8*, 2380–2391.

Martin, G., Keller, W., and Doublie, S. (2000). Crystal structure of mammalian poly(A) polymerase in complex with an analog of ATP. EMBO J. *19*, 4193–4203.

McManus, M.T., Adler, B.K., Pollard, V.W., and Hajduk, S.L. (2000). *Trypanosoma brucei* guide RNA poly(U) tail formation is stabilized by cognate mRNA. Mol. Cell. Biol. *20*, 883–891.

McManus, M.T., Shimamura, M., Grams, J., and Hajduk, S.L. (2001). Identification of candidate mitochondrial RNA editing ligases from *Trypanosoma brucei*. RNA *7*, 167–175.

Milchev, G.I., and Hadjiolov, A.A. (1978). Association of poly(A) and poly(U) polymerases with cytoplasmic ribosomes. Eur. J. Biochem. *84*, 113–121.

Militello, K.T., and Read, L.K. (2000). UTP-dependent and -independent pathways of mRNA turnover in *Trypanosoma brucei* mitochondria. Mol. Cell. Biol. *20*, 2308–2316.

Missel, A., Souza, A.E., Norskau, G., and Goringer, H.U. (1997). Disruption of a gene encoding a novel mitochondrial DEAD-box protein in *Trypanosoma brucei* affects edited mRNAs. Mol. Cell. Biol. *17*, 4895–4903.

Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA *95*, 14687–14692.

Panigrahi, A.K., Gygi, S.P., Ernst, N.L., Igo, R.P., Palazzo, S.S., Schnaufer, A., Weston, D.S., Carmean, N., Salavati, R., Aebersold, R., and Stuart, K.D. (2001a). Association of two novel proteins, TbMP52 and TbMP48, with the *Trypanosoma brucei* RNA editing complex. Mol. Cell. Biol. *21*, 380–389.

Panigrahi, A.K., Schnaufer, A., Carmean, N., Igo, R.P., Jr., Gygi, S.P.,

Ernst, N.L., Palazzo, S.S., Weston, D.S., Aebersold, R., Salavati, R., and Stuart, K.D. (2001b). Four related proteins of the *Trypanosoma brucei* RNA editing complex. Mol. Cell. Biol. *21*, 6833–6840.

Peris, M., Simpson, A.M., Grunstein, J., Liliental, J.E., Frech, G.C., and Simpson, L. (1997). Native gel analysis of ribonucleoprotein complexes from a *Leishmania tarentolae* mitochondrial extract. Mol. Biochem. Parasitol. *85*, 9–24.

Rusche, L.N., Cruz-Reyes, J., Piller, K.J., and Sollner-Webb, B. (1997). Purification of a functional enzymatic editing complex from *Trypanosoma brucei* mitochondria. EMBO J. *16*, 4069–4081.

Rusché, L.N., Huang, C.E., Piller, K.J., Hemann, M., Wirtz, E., and Sollner-Webb, B. (2001). The two RNA ligases of the *Trypanosoma brucei* RNA editing complex: cloning the essential band IV gene and identifying the band V gene. Mol. Cell. Biol. *21*, 979–989.

Sabatini, R., and Hajduk, S.L. (1995). RNA ligase and its involvement in guide RNA/mRNA chimera formation. J. Biol. Chem. *270*, 7233–7240.

Sachs, A., and Wahle, E. (1993). Poly(A) tail metabolism and function in eucaryotes. J. Biol. Chem. *268*, 22955–22958.

Schnaufer, A., Panigrahi, A.K., Panicucci, B., Igo, R.P., Salavati, R., and Stuart, K. (2001). An RNA ligase essential for RNA editing and survival of the bloodstream form of *Trypanosoma brucei*. Science *291*, 2159–2161.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K., Plasterk, R.H.A., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465–476.

Simpson, L., Wang, S.H., Thiemann, O.H., Alfonzo, J.D., Maslov, D.A., and Avila, H.A. (1998). U-insertion/deletion edited sequence database. Nucleic Acids Res. *26*, 170–176.

Trippe, R., Sandrock, B., and Benecke, B.J. (1998). A highly specific terminal uridylyl transferase modifies the 3′-end of U6 small nuclear RNA. Nucleic Acids Res. *26*, 3119–3126.

Vanhamme, L., Perez-Morga, D., Marchal, C., Speijer, D., Lambert, L., Geuskens, M., Alexandre, S., Ismaïli, N., Göringer, U., Benne, R., and Pays, E. (1998). *Trypanosoma brucei* TBRGG1, a mitochondrial oligo(U)-binding protein that colocalizes with an in vitro RNA editing activity. J. Biol. Chem. *273*, 21825–21833.

Wang, Z.F., Morris, J.C., Drew, M.E., and Englund, P.T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. *275*, 40174–40179.

White, T., and Borst, P. (1987). RNA end-labeling and RNA ligase activities can produce a circular ribosomal RNA in whole-cell extracts from trypanosomes. Nucleic Acids Res. *15*, 3275–3290.

Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A. (1999). A tightly regulated inducible expression system for conditional gene knockouts and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *99*, 89–101.

Zabel, P., Dorssers, L., Wernars, K., and Van Kammen, A. (1981). Terminal uridylyl transferase of vigna unguiculata: purification and characterization of an enzyme catalyzing the addition of a single UMP residue to the 3′-end of an RNA primer. Nucleic Acids Res. *9*, 2433–2453.

Accession Numbers

The GenBank Accession numbers for the sequences in this paper are AY029069 and AY029070 for *L. tarentolae* and *T. brucei* TUTase, respectively.