THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 40, pp. 29073–29080, October 5, 2007

Uridylate-specific 3′–5′-Exoribonucleases Involved in Uridylate-deletion RNA Editing in Trypanosomatid Mitochondria*

Received for publication, June 4, 2007, and in revised form, August 13, 2007 Published, JBC Papers in Press, August 15, 2007, DOI 10.1074/jbc.M704551200

Kestrel Rogers, Guanghan Gao[1], and Larry Simpson[2]

From the Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California 90095

In kinetoplastid protists, maturation of mitochondrial pre-mRNAs involves the insertion and deletion of uridylates (Us) within coding regions, as specified by mitochondrial DNA-encoded guide RNAs. U-deletion editing involves endonucleolytic cleavage of the pre-mRNA at the editing site followed by U-specific 3′–5′-exonucleolytic removal of nonbase-paired Us prior to ligation of the two mRNA cleavage fragments. We showed previously that an exonuclease/endonuclease/phosphatase (EEP) motif protein from *Leishmania major,* designated RNA editing exonuclease 1 (REX1) (Kang, X., Rogers, K., Gao, G., Falick, A. M., Zhou, S.-L., and Simpson, L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 1017–1022), exhibits 3′–5′-exonuclease activity. Two EEP motif proteins have also been identified in the *Trypanosoma brucei* editing complex. *Tb*REX1 is a homologue of *Lm*REX1, and *Tb*REX2 shows homology to another editing protein in *L. major,* which lacks the EEP motif (*Lm*REX2*). Here we have expressed the *T. brucei* EEP motif proteins in insect cells and purified them to homogeneity. We showed that these are U-specific 3′–5′-exonucleases that are inhibited by base pairing of 3′ Us. The recombinant EEP motif alone also showed 3′–5′ U-specific exonuclease activity, and mutations of the REX EEP motifs greatly reduced exonuclease activity. The absence of enzymatic activity in *Lm*REX2* was confirmed with a purified recombinant protein. We showed that pre-cleaved U-deletion editing could be reconstituted with either *Tb*REX1 or *Tb*REX2 in combination with either RNA ligase, *Lm*REL1, or *Lm*REL2. Down-regulation of *Tb*REX2 expression by conditional RNA interference had little effect on parasite viability or sedimentation of the L-complex, suggesting either that *Tb*REX2 is inactive *in vivo* or that *Tb*REX1 can compensate for the loss of *Tb*REX2 function in down-regulated cells.

The majority of mitochondrial pre-mRNAs in kinetoplastid protists are edited through the insertion and deletion of uridylate residues (Us).[3] The editing process is directed by guide RNAs (gRNAs) that initially anneal to the pre-edited mRNA at the first downstream editing site via a region of complementarity termed the "anchor sequence." An editing cycle involves an enzyme cascade (2) initiated by specific RNA editing endonucleases (REN), one of which (REN1) cleaves the mRNA immediately 5′ to the mRNA/gRNA duplex at U-deletion sites and the other (REN2) cleaves at U-insertion sites (3–5). The upstream gRNA sequence directs the modification of the 3′ end of the 5′ mRNA fragment by either the insertion of complementary Us by an RNA editing terminal uridylyltransferase (RET2) or by the removal of nonbase-paired Us by an RNA editing exonuclease (REX). Ligation of the mRNA fragments by an RNA editing ligase (REL) terminates the single site editing cycle. Editing then proceeds to the adjacent upstream site until all sites mediated by the single gRNA are edited resulting in an edited mRNA/gRNA duplex. Multiple overlapping gRNAs are required for extensive editing (pan-editing). Editing is catalyzed by a ribonucleoprotein complex (editosome) consisting of the RNA ligase-containing complex (L-complex) together with several additional complexes involved with RNA binding and 3′ U addition to the gRNAs linked by bound RNA (6).

The current understanding of U-insertion/deletion RNA editing encompasses data derived from both *Trypanosoma brucei* and *Leishmania tarentolae* model systems. Up to 20 L-complex components have been identified experimentally in *T. brucei* (7) and *L. tarentolae* (8), and orthologues of L-complex components have been identified in the *Leishmania major* genome (7, 8). Enzymatic activities consistent with the enzyme cascade model have been confirmed *in vitro* for several components of the L-complex, including REL1, REL2 (9–11), RET2 (12, 13), and REN1 (5). Identified activities were similar in orthologues from both *Leishmania* and *Trypanosoma*. REX1 activity has also been confirmed *in vitro* but only with the *L. major* orthologue (1).

*Lm*REX1 was first identified as a candidate exonuclease by the presence of a conserved exonuclease/endonuclease/phosphatase (EEP, Pfam accession number PF03372) domain (6). We subsequently found recombinant *Lm*REX1

* This work was supported by National Institutes of Health Grant AI09102 (to L. S.) and by Ruth L. Kirschstein National Research Service Award GM07185 (to K. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Present address: Kurland Heart Institute, 200 Med Plaza, Los Angeles, CA 90095.

[2] To whom correspondence should be addressed: David Geffen School of Medicine at UCLA, 1602 Molecular Science Bldg., Los Angeles, CA 90095-1489. Fax: 310-341-2271; E-mail: simpson@kdna.ucla.edu.

[3] The abbreviations used are: Us, uridylate residues; REX, RNA editing exonuclease; EN, RNA editing endonuclease; REEL, RNA editing RNA ligase; RET, RNA editing terminal uridylyltransferase; EEP, exonuclease/endonuclease/phosphatase motif; TAP, tandem affinity purification; gRNA, guide RNA; cbp, calmodulin-binding peptide; TEV, tobacco etch virus; RNAi, RNA interference; dsRNA, double-stranded RNA; r, recombinant.

to be a 3′–5′ U-specific exonuclease (1). However, the *T. brucei* L-complex contains a second EEP domain protein (14–17), which we designated REX2 for its putative function (1). *Tb*REX2 is a component of the REL1 subcomplex of the L-complex that is competent for "pre-cleaved" deletion editing *in vitro* (18). Surprisingly, the *Leishmania* orthologue of *Tb*REX2 lacks an EEP domain, suggesting an absence of exonuclease activity, and we have named the *Leishmania* orthologue REX2*. Here we further investigate the kinetoplastid REX proteins by purification and enzymatic characterization of recombinant REX proteins from *T. brucei*, and we demonstrate for the first time a difference in enzymatic content of the *Leishmania* and *Trypanosoma* L-complexes.

EXPERIMENTAL PROCEDURES

Cloning of REX Proteins into Baculovirus Expression Vectors—*Tb*REX1, *Tb*REX2, and *Lm*REX2* were amplified from *T. brucei* and *L. major* genomic DNA using the respective primer pairs 5′-**GGATCC**AAGCTTATGGCATTGGCTCAGTCATG-3′, 5′-AAGCTTCTA**TCTAGA**AAGGGCACCAATAATCTGAACTTCCG-3′; 5′-**GGATCC**AAGCTTATGTTGCGCCGCAGTCGCTT-3′, 5′-AAGCTTCTA**TCTAGA**AACCACCTGAAACTCAACAAGGAGACCG-3′; and 5′-CG**GGATCC**ATGTACGGCGTGACAC-3′, 5′-AAGCTT**TCTAGA**GCCGGGCATGCCACT-3′ and cloned into pCR2.1-TOPO (Invitrogen). Restriction sites are indicated in boldface type. Genes were released with BamHI and XbaI and inserted into the corresponding sites of a modified pFastBac1 vector (Invitrogen) containing C-terminal tandem affinity purification (TAP) (calmodulin-binding peptide (cbp), TEV protease site, protein A) epitope tags to generate pFastBac1-*Lm*REX2*-TAP, pFastBac1-*Tb*REX1-TAP, and pFastBac1-*Tb*REX2-TAP vectors (9). Double amino acid mutations were made in the *Tb*REX1 and *Tb*REX2 EEP domains using the QuickChange site-directed mutagenesis kit (Stratagene) and the primers 5′-GCATATTTGTTCCCATCCG**CTA**ACTACGGCGTAAGTATA-3′ and 5′-GAGTATTTGTACCCGTCTG**CCA**ATTTCGGTCTCCTTGTTG-3′ (mutations indicated in boldface) to generate the plasmids pFastBac1-*Tb*REX1(D881A,H882N)-TAP and pFastBac1-*Tb*REX2(D896A,H897N)-TAP respectively.

Expression and Purification of LmREX2, TbREX1, and TbREX2*—TAP-tagged *Lm*REX2*, *Tb*REX1, *Tb*REX1(D881A, H882N), *Tb*REX2, and *Tb*REX2(D896A,H897N) were expressed in *Spodoptera frugiperda* (Sf9) cells. DH10Bac cells (Invitrogen) were transformed with pFastBac1 fusion protein constructs for transposition into Bacmid shuttle vectors. Sf9 cells were maintained in Sf900II (Invitrogen) or CCM3 media (HyClone) and were transfected with shuttle vector DNA using Cellfectin (Invitrogen) to generate recombinant baculovirus stocks. Sf9 cultures at 1×10^6 cells/ml were infected with recombinant baculovirus for 48 h. Optimal virus titer used to initiate expression was determined empirically.

r*Lm*REX2*-cbp, r*Tb*REX1-cbp, r*Tb*REX1(D881A,H882N)-cbp, r*Tb*REX2-cbp, and r*Tb*REX2(D896A,H897N)-cbp were purified from total cell lysates of baculovirus infected cells. Tandem affinity purification was performed as described previously (6). Affinity-purified protein was diluted into 50 mM phosphate buffer, pH 7.9, loaded onto a Mono S 5/50 GL column (Amersham Biosciences), and eluted over a 0–1 M NaCl gradient. Fractions containing recombinant protein were concentrated by ultrafiltration in Ultrafree centrifugal filter units (10-kDa nominal molecular weight limit, Millipore) and applied to a Superdex 200 10/300 GL column (Amersham Biosciences) equilibrated with 50 mM phosphate and 300 mM NaCl. Purity of protein preparations was monitored by SDS-PAGE in 8–16% acrylamide Novex gels (Invitrogen) stained with SYPRO Ruby (Molecular Probes). Purified protein was stored at −80 or at −20 °C in 50% glycerol (v/v). Protein concentration was determined in-gel by comparison to pre-diluted bovine serum albumin protein assay standards (Pierce).

Cytosolic Expression of the TbREX1 EEP Domain—A *Tb*REX1 gene fragment coding for amino acids 606–902 was PCR-amplified using the primer pair 5′-**GGATCC**ATGGCAAAAGAGGTT-3′ and 5′-AAGCTTCTA**TCTAGA**AAGGGCACCAATAATCTGAACTTCCG-3′ and cloned into pCR2.1-TOPO. A modified TAP tag containing a protein C epitope instead of cbp was PCR-amplified from the pC-PTP-NEO vector (19) using the primers 5′-TCTAGAGGCTCCGGCTCCATGGGAAGATCAGGTGGAT-3′ and 5′-GCGGCCGCTCAGGTTGACTTCCC-3′ and after digestion with XbaI and NotI was inserted into the corresponding sites of the *Lt*REL1-TAP (6) episomal expression vector, replacing the cbp tag. An internal BamHI site was removed from the protein C epitope using the QuickChange site-directed mutagenesis kit and the primer 5′-CCGGCTCCATGG**AA**GAAGATCAGGTGGA**C**CCTCGTCTTATTGATG-3′. The *Tb*REX1-(606–902) sequence was released from pCR2.1-TOPO with BamHI and XbaI and inserted into the corresponding sites of the modified TAP vector. *L. tarentolae* UC strain cells were maintained in BHI media (Difco) supplemented with 10 mg/ml hemin-HCl. Cells were electroporated with the TAP vector and selected for retention of plasmid by plating on agarose in the presence of 200 μg/ml G418. Transformed cells were maintained in 100 μg/ml G418 (20). Affinity purification of *Tb*REX1-(606–902)-protein C was performed as described previously (19).

*Tandem Affinity Purification of LmREX2**—L-complex containing TAP-tagged *Lm*REX2* was purified from *L. tarentolae*. *Lm*REX2* released from pCR2.1-TOPO-*Lm*REX2* with BamHI and XbaI and inserted into the corresponding sites of *Lt*REL1-TAP (6) to generate *Lm*REX2*-TAP vector for transformation of cells was conducted as described above. Tandem affinity purification of L-complex was performed as described previously (6).

Exonuclease Activity of Recombinant REX Proteins—Purified proteins were tested for exonuclease activity with the following synthetic RNAs: 6U, 5′-GCUAUGUCUGCUAACUUGUUUUUU-3′; 6G, 5′-GCUAUACAUGCUACAUUGGGGGG-3′; 6C, 5′-GCUAUGUCUGCUAACUUGCCCCCC-3′; 6A, 5′-GCUAUGUCUGCUAACUUGAAAAAA-3′; and 1U, 5′-GCUAUGUCUGCUAACUUGUAUCGU-3′. RNAs were 5′-radiolabeled with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]ATP. In 10-μl reactions 1 pmol of 5′-labeled RNA was diluted into a standard 10-μl reaction containing 5 mM $MgCl_2$, 10 mM Tris-hydrochloride, pH 7.8, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin. Either 28 fmol of r*Tb*REX1 or 54 fmol of r*Tb*REX2 were added to reactions and incubated at 27 °C from 1 to 16 min before

termination of reactions by the addition of urea-saturated formamide. r*Lm*REX2* activity was tested by varying the concentration of recombinant protein from 0.01 to 1 μM in 32-min reactions. Activity of r*Tb*REX mutants was assayed against 1U RNA substrate in time course reactions containing 53 fmol of either r*Tb*REX1 or r*Tb*REX1(D881A,H882N) or 152 fmol of either r*Tb*REX2 or r*Tb*REX2(D896A,H897N). Reaction products were resolved by denaturing PAGE in 8 M urea, 15% acrylamide gels. Dried gels were exposed to storage phosphor screens for visualization by PhosphorImager.

Cloning of RNAi Vector and Down-regulation of TbREX2—A 500-bp fragment of the *Tb*REX2 gene was PCR-amplified using the primers 5′-**GGATCCAAGCTT**ATGTTGCGCCGCAGTCGCTT-3′ and 5′-**GGATCCTCTAGA**CGAGGTCAAGCATCCACGCA-3′ and cloned into pCR2.1-TOPO. The gene fragment was released first with HindIII and XbaI and subsequently with BamHI for insertion into the corresponding sites of the pLew100-HX-GFP vector as inverted copies separated by a spacer element (21). The fragment was also released with BamHI and HindIII and inserted into the corresponding sites of the p2T7^{Ti}-177 vector (22). RNAi vectors were linearized with NotI and electroporated into strain 29-13 *T. brucei* procyclic form cells that express T7 RNA polymerase and tetracycline repressor. Procyclic cells were maintained in SDM79 media supplemented with 15% fetal bovine serum, 15 μg/ml G418, and 50 μg/ml hygromycin. After electroporation, cultures were selected for genomic integration of the construct at the rDNA spacer (pLew100-HX-GFP) or the 177-bp satellite (p2T7^{Ti}-177) by plating on agarose in the presence of 2.5 μg/ml phleomycin (23). Transcription of *Tb*REX2 dsRNA was induced by addition of 1 μg/ml tetracycline.

For growth curve analysis, cells were maintained in log phase by dilution to 10^6 cells/ml every 48 h. Mitochondria were isolated from log phase cultures as described (24). Extracts from 100 mg of purified mitochondria were prepared by sonication in 50 mM HEPES, pH 7.9, 60 mM KCl, 10 mM $MgCl_2$, and 0.5% Nonidet P-40. Extracts were clarified by centrifugation for 30 min at 70,000 rpm in a Beckman TLA-100.4 rotor and sedimented in a 10–30% glycerol gradient for 20 h at 30,000 rpm in a Beckman SW41Ti rotor. Gradient fractions were auto-adenylated for 20 min at 27 °C with 5 μCi of [α-^{32}P]ATP per 10 μl and resolved by SDS-PAGE on 8–16% acrylamide NOVEX gels (Invitrogen) prior to transfer to Protran BA83 nitrocellulose (Schleicher & Schuell) for immunodetection or autoradiography. *Tb*REX2 mRNA levels were determined by reverse transcription-PCR analysis. Total RNA was purified and DNase treated using the RNeasy mini kit (Qiagen). SuperScript II reverse transcriptase (Invitrogen) was used for cDNA synthesis. The primer pairs 5′-TGATCATGTCGCCGTCGACG-3′, 5′-TTGAAGTTCACGCCCAGGCC-3′; and 5′-TGACTACGTTGCGGTGGACGCC-3′, 5′-CTCGCGGTCGCGCTTTGAGA-3′ were used to PCR-amplify regions of *Tb*REX2 and *Tb*REX1 respectively.

Kinetic Analysis of TbREX1 and TbREX2 Activities—r*Tb*REX1 and r*Tb*REX2 were incubated with 5′ end-labeled 1U substrate RNA in standard reaction buffer. To determine the linear range of product accumulation, time course reactions at various REX protein concentrations were monitored by denaturing PAGE. Kinetic parameters were determined by incubating 22 fmol of r*Tb*REX1 or 54 fmol of r*Tb*REX2 in 10-μl reactions with varying concentrations of 1U substrate for 10 and 15 min, respectively. Substrate concentration ranged from 50 nM to 1.5 μM. The percent of substrate cleavage visualized by PhosphorImager was quantified using ImageQuant (version 5.2, GE Healthcare) and used to calculate enzyme velocity (fmol/min). Kinetic parameters were calculated from nonlinear regression of velocity *versus* substrate concentration plots using GraphPad Prism (version 4.01, GraphPad Software, Inc.).

Pre-cleaved RNA Editing Assays—The following synthetic RNAs were used in pre-cleaved editing assays: 5′ UU fragment, 5′-GCACUACACGAUAAAUAUAAAAAGUU-3′; 3′ fragment, 5′-AACAUUAUGCUUCUUCGddC-3′; −0 gRNA, 5′-AAGAAGCAUAAUGUUagCUUUUUAUAUUUAUCGUGUAGUCddG-3′ (guiding nucleotides in lowercase); −1gRNA, 5′-AAGAAGCAUAAUGUUaCUUUUUAUAUUUAUCGUGUAGUCddG-3′ (guiding nucleotide in lowercase); −2 gRNA, 5′-AAGAAGCAUAAUGUUCUUUUUAUAUUUAUCGUGUAGUCddG-3′.

Reactions contained 1 pmol of kinase-labeled 5′ UU fragment, 2 pmol of 3′ fragment, and 4 pmol of a single gRNA. RNAs were annealed by heating to 70 °C and slowly cooling to 4 °C prior to addition of reaction buffer. Recombinant *Tb*REL1 and *Tb*REL2 purified from Sf9 cells were added to some reactions (9).

RESULTS

Expression and Purification of the T. brucei and L. major REX Proteins—PFAM analysis of the *Tb*REX1, *Tb*REX2, and *Lm*REX1 amino acid sequences indicated the presence of the EEP motif in all three sequences (Fig. 1*A*), as demonstrated previously. Also as noted previously, the *Lm*REX2 homologue sequence lacks any vestige of this motif, leading us to use the *Lm*REX2* nomenclature for this protein. As discussed in the Introduction, this finding raises a paradox because the REL1 subcomplex of the L-complex in *T. brucei* and *L. tarentolae*, which has been proposed to be mainly involved in U-deletion editing, appears to contain only REX2 (6, 18). To address the possible functional role of *Lm*REX2*, we decided to analyze a recombinant *Lm*REX2* protein in addition to recombinant *Tb*REX1 and *Tb*REX2 proteins. The purification to homogeneity of insect cell-expressed TAP-tagged recombinant *Tb*REX1, *Tb*REX2, and *Lm*REX2* proteins by successive affinity and chromatographic steps is shown in Fig. 1*B*.

Exonuclease Activity of Recombinant REX Proteins—The recombinant *Tb*REX1 and *Tb*REX2 proteins were found to exhibit a robust 3′–5′-exonuclease activity (Fig. 2, *A* and *B*). This activity was specific for 3′ uridylates, and no degradation was observed with RNAs terminating in 3′ A, G, or C residues. This exouridylase activity appeared distributive in nature. A processive type activity was observed using only affinity-purified proteins (data not shown). The r*Tb*REX activity was dependent on magnesium cation and was sensitive to chelating agents (Fig. 2*C*).

On the other hand, the recombinant *Lm*REX2* did not exhibit detectable 3′–5′-exonuclease activity against any substrate RNA tested (Fig. 2*D*). No exonuclease activity was

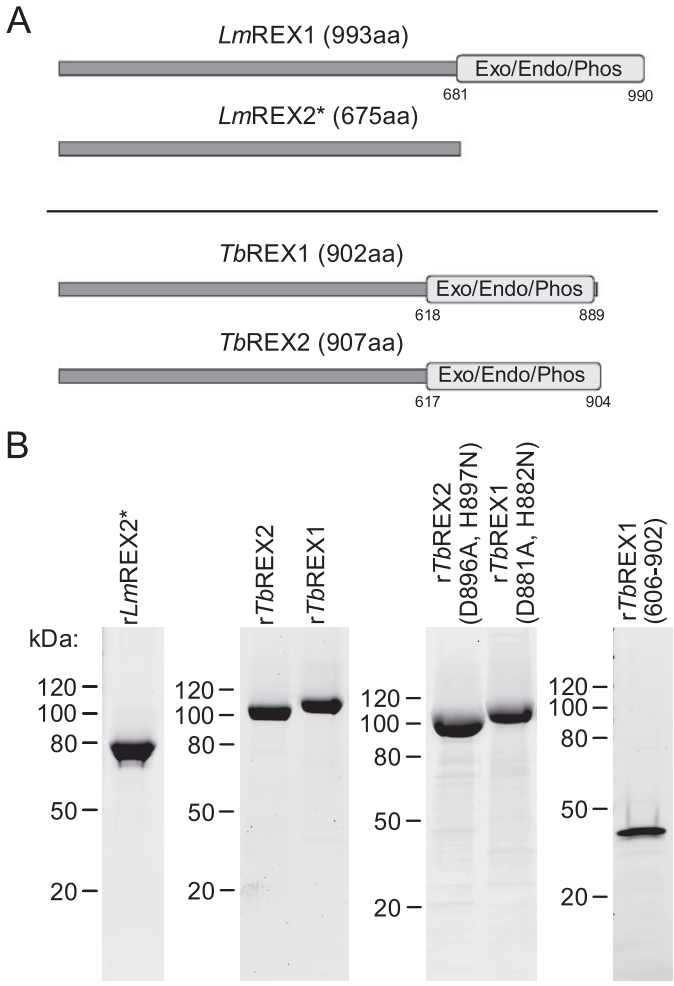

FIGURE 1. **Expression and purification of r*Tb*REX1, r*Tb*REX2, and r*Lm*REX2*.** *A,* EEP motif proteins of *Leishmania* and *Trypanosoma.* EEP domains were predicted by PFAM analysis. The *Leishmania* REX2 orthologue lacks an EEP motif. A majority of proteins were expressed in insect cells as TAP tag fusions. Proteins were purified by sequential affinity, cation exchange, and gel filtration steps. r*Tb*REX1-(606–902) was expressed in the cytosol of *L. tarentolae* as a modified TAP tag fusion containing protein C instead of cbp. r*Tb*REX1-(606–902) was purified by sequential affinity chromatography. *B,* purity was assessed by SYPRO Ruby staining after SDS-PAGE in 8–16% acrylamide gels. Molecular weight markers are indicated for each gel.

observed even when the r*Lm*REX2* concentration was increased by 2 orders of magnitude *versus* the concentrations of r*Tb*REX1 and r*Tb*REX2 used previously. The presence of *Lt*REX2* in the L-complex was demonstrated by transfecting *L. tarentolae* cells with TAP-tagged *Lm*REX2* and showing that the TAP pulldown had an identical polypeptide profile as L-complex isolated by use of known L-complex components (data not shown).

Down-regulation of TbREX2 Expression Has a Minor Effect on Cell Growth—*Tb*REX2 gene expression was down-regulated by tetracycline-inducible RNA interference. A procyclic stage *T. brucei* cell line was generated in which transcription of stem-loop RNA from inverted copies of a 500-bp *Tb*REX2 gene fragment was driven by a tetracycline-inducible PARP promoter. Induction of RNAi for 3 days resulted in reduction of *Tb*REX2 mRNA levels as detected by reverse transcription-PCR from total RNA (Fig. 3*A*, *right panel*). Loss of *Tb*REX2 expression had little effect on parasite viability and growth (Fig. 3*A*, *left panel*).

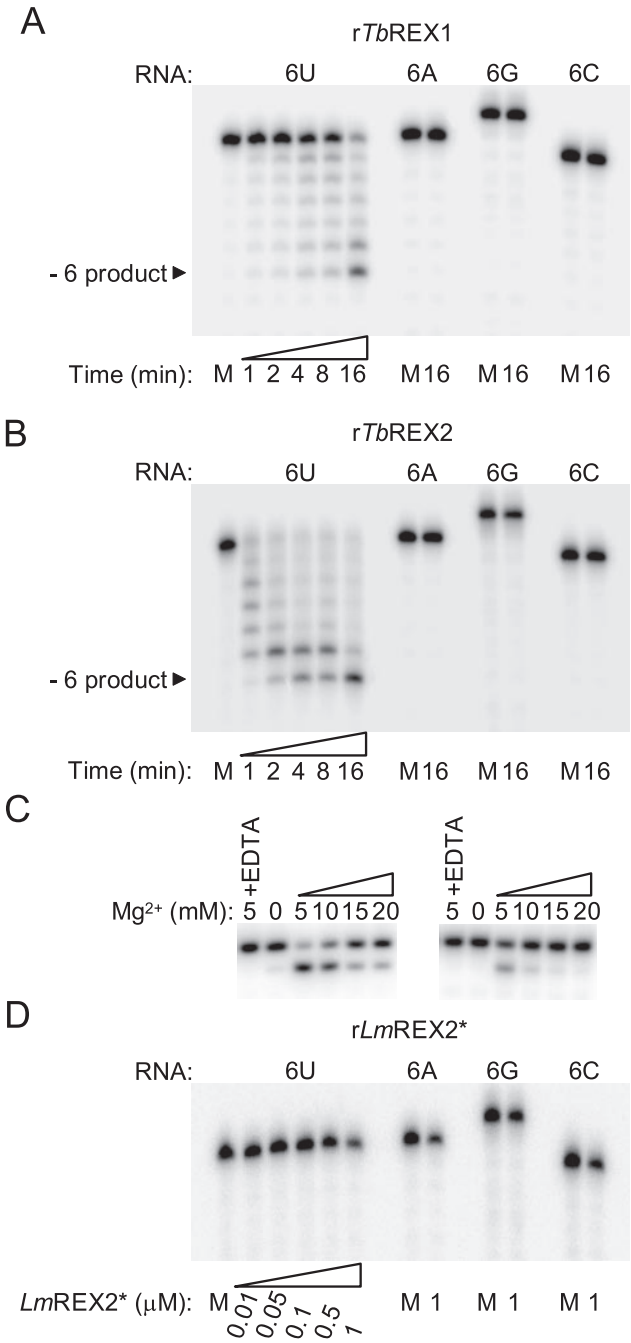

FIGURE 2. **r*Tb*REX1 and r*Tb*REX2 are 3′–5′ U-specific exonucleases, whereas r*Lm*REX2* appears inactive.** r*Tb*REX1 (2.8 nM) (*A*) or r*Tb*REX2 (5.4 nM) (*B*) were incubated with 5′ ^{32}P-labeled single-stranded RNA substrates 3′ terminating in 6 Us, As, Gs, or Cs. At the indicated time points, reaction products were separated by 8 M urea, 15% acrylamide denaturing PAGE, and dried gels were visualized with PhosphorImager screens. Mock reactions (*M*) did not include enzyme. *C,* r*Tb*REX1 (*left panel*) and r*Tb*REX2 (*right panel*) require magnesium for activity against 1U RNA with a single 3′ uridylate. *D,* r*Lm*REX2* was assayed after 32 min of incubation for nuclease activity at increasing protein concentrations.

However, after 5 days of induction a slight but consistent reduction in growth rate was observed. An alternate RNAi cell line expressing *Tb*REX2 dsRNA from opposing T7 RNA polymerase promoters exhibited no growth phenotype after RNAi induction (data not shown). This behavior contrasts to the previously reported strong effect of down-regulation of *Tb*REX1 expression on cell growth (1).

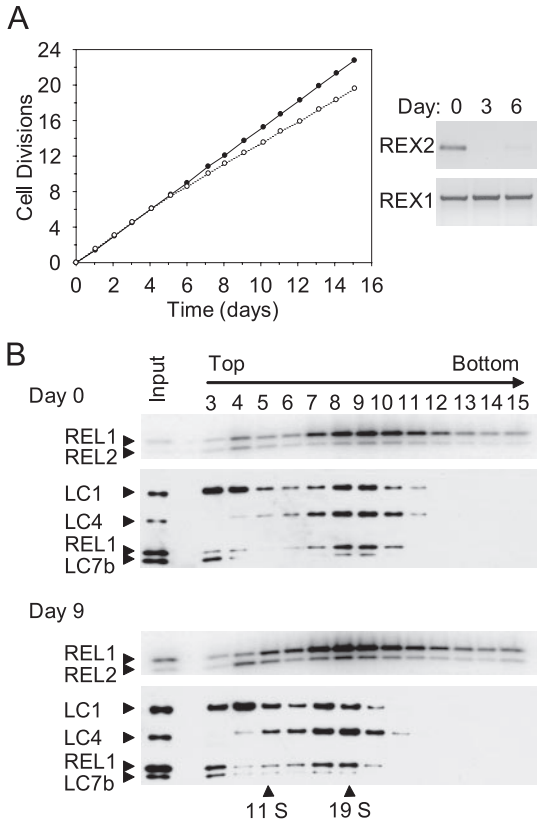

FIGURE 3. **RNAi down-regulation of *Tb*REX2 expression.** Expression of *Tb*REX2 dsRNA in *T. brucei* 29-13 procyclic cells was induced at day 0. *A, left panel*, growth curves of induced (○) and uninduced (●) cells maintained in log phase growth by periodic dilution. *A, right panel*, reverse transcription-PCR of *Tb*REX2 and *Tb*REX1 total cell RNA showed the absence of *Tb*REX2 RNA after 3 days of RNAi induction. *B,* sedimentation of mitochondrial lysates before and after 9 days of RNAi induction. Lysates were sedimented on 10–30% glycerol gradients for 20 h in an SW41 rotor. Gradient fractions were labeled by auto-adenylation to detect editing ligases (day 0 and day 9, *upper panels*) and resolved by SDS-PAGE. *Tb*LC1, *Tb*LC4, *Tb*REL1, and *Tb*LC7b were detected by Western blotting (day 0 and day 9, *lower panels*).

The effect of *Tb*REX2 down-regulation on L-complex stability was assayed by sedimentation of mitochondrial lysate before and after 9 days of RNAi induction. Only minor changes in L-complex sedimentation were apparent (Fig. 3*B*), suggesting that *Tb*REX2 is not required to maintain the stability or the structure of the L-complex. In addition, no major differences were detected among the distribution of the L-complex components LC1 (MP81), LC4 (MP63), LC7b (MP42), and REL2. The increased level of REL1 detected in fraction three after RNAi is consistent with previous findings that suggest the presence of a REL1 subcomplex consisting of REX2, REL1, and LC4 (18). REX2 may help stabilize the interaction of REL1 with the L-complex.

REX EEP Domains Are Required for Exonuclease Activity—The EEP domains from diverse proteins share several highly conserved amino acids (25), which are also conserved in the *Tb*REX1 and *Tb*REX2 EEP domains (Fig. 4*A*) (26, 27). To confirm that the ribonuclease activity of both *Tb*REX1 and *Tb*REX2 is because of the EEP domains, *Tb*REX proteins mutated in the conserved EEP motif amino acids were tested for exonuclease activity *in vitro*. Two amino acids were mutated in both *Tb*REX1(D881A,H882N), and *Tb*REX2(D896A,H897N), and the mutant proteins were purified after overexpression in insect cells (Fig. 1*B*). The exonuclease activity of the mutant proteins was evaluated with a single-stranded RNA substrate terminating in a single 3′ uridylate. Time course analysis of both wild type and mutant protein reactions showed decreased activity of mutant *versus* wild type REX proteins (Fig. 4*B*).

The role of the EEP motif in catalysis was further examined through expression of the *Tb*REX1 C-terminal EEP domain. A truncated *Tb*REX1 peptide containing amino acids 606–902 was purified after overexpression in the cytosol of *L. tarentolae*. r*Tb*REX1-(606–902) was sufficient for 3′–5′-exonuclease activity, and the exonuclease activity was U-specific (Fig. 4*C*). Our previous observation that, during the purification of *Lm*REX1, C-terminal proteolytic fragments co-fractionated with a nonspecific exonuclease activity (1) could represent contamination with nonspecific nucleases, conformational changes induced by proteolysis, or authentic differences in the structural stability and determinants of specificity for EEP domains from *L. major* and *T. brucei*.

Kinetic Analysis of REX 3′–5′-Exonuclease Activity—Steady state kinetics of the *Tb*REX exonuclease reactions were determined using a single-stranded RNA substrate terminating in a single 3′ U residue. The r*Tb*REX1 and r*Tb*REX2 reactions yielded similar apparent K_m values of 536.1 ± 84.99 and 224.5 ± 25.83 nM, indicating a high affinity of these enzymes for the RNA substrate (Fig. 5, *A* and *B*). The catalytic efficiencies (k_{cat}/K_m) were 1.7×10^4 and 6.2×10^3 s^{-1} M^{-1}, respectively. The exonuclease efficiency of *Tb*REX1 was 2.7-fold higher than that of *Tb*REX2; however, the similarity of the two values suggests that both enzymes may exhibit functionally relevant catalytic activity *in vivo*.

REX1 and REX2 Exouridylase Activity Is Affected by gRNA Sequence—Although the *Tb*REX enzymes exhibit similar activities against simple substrates *in vitro*, the REX proteins may exhibit alternate specificities on complex substrates such as editing sites *in vivo*. To simulate substrate conditions at U-deletion editing sites, r*Tb*REX enzymes were tested for activity against pre-cleaved RNA substrates that resemble deletion editing sites after an initial endonucleolytic cleavage step (28). Both enzymes were capable of 3′–5′-exonucleolytic removal of unpaired Us from the 3′ end of the 5′ mRNA fragment (Fig. 6*A*). Both enzymes also exhibited a preference for single-stranded 3′ Us; trimming of the 3′-terminal Us stopped to a large extent at duplex regions generated by complementary gRNAs (Fig. 6, *A* and *B*). The partial degradation of base-paired 3′ Us may be due to limited breathing of the annealed substrate RNAs.

Both REX1 and REX2 Remove Us from Pre-cleaved Deletion Substrates and Can Be Used to Reconstitute Pre-cleaved U-deletion RNA Editing in Vitro—We have shown previously that pre-cleaved deletion editing can be reconstituted using the *Leishmania* proteins, r*Lm*REX1 and r*Lm*REL1 (1). Full round single site deletion editing can be reconstituted by the further addition of the U-deletion site-specific endonuclease, r*Lm*MP90 (r*Lm*REN2) (5). Recombinant *Tb*REX1 and *Tb*REX2 were combined with recombinant *Tb*REL1 and *Tb*REL2 RNA ligases (9) that were purified from overexpression in insect cells and incubated with the −2U pre-cleaved substrate. Any combination of REX and REL proteins was sufficient to generate fully edited products (Fig. 6*B*). This is in marked contrast to the *Leishmania* system in which the r*Lm*REX1 and r*Lm*REL1 com-

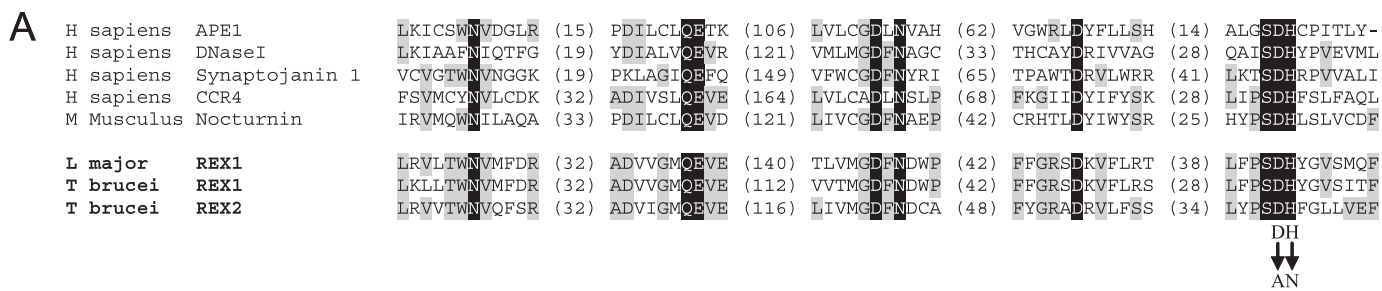

FIGURE 4. **REX exonuclease activity is because of the presence of the EEP domain.** *A,* partial sequence alignment of REX EEP domains and representative EEP proteins reveals conservation of the five motifs used to identify EEP domains. Sequences were aligned with the Pfam EEP Hidden Markov model using HMMalign. Mutation of EEP domain residues reduces REX exonuclease activity. *B, left panel*, double mutant (□) r*Tb*REX1-D881A,H882N; and *B, right panel*, r*Tb*REX2-D896A,H897N proteins were compared with wild type (■) r*Tb*REX enzymes in time course assays with 5′-radiolabeled 1U RNA substrate. Reaction products were resolved by denaturing 8 M urea 15% acrylamide gel electrophoresis. Percent of substrate converted to the −1 product was calculated by ImageQuant (Amersham Biosciences) analysis of PhosphorImager scanned gels. *C, Tb*REX1 EEP domain is sufficient for U-specific exonuclease activity. A truncated r*Tb*REX1-(606–902) protein containing only the *Tb*REX1 C-terminal EEP domain was incubated with 5′-radiolabeled RNA substrates 3′ terminating in 6 Us, As, Gs, or Cs. Mock reactions (*M*) without enzyme are indicated.

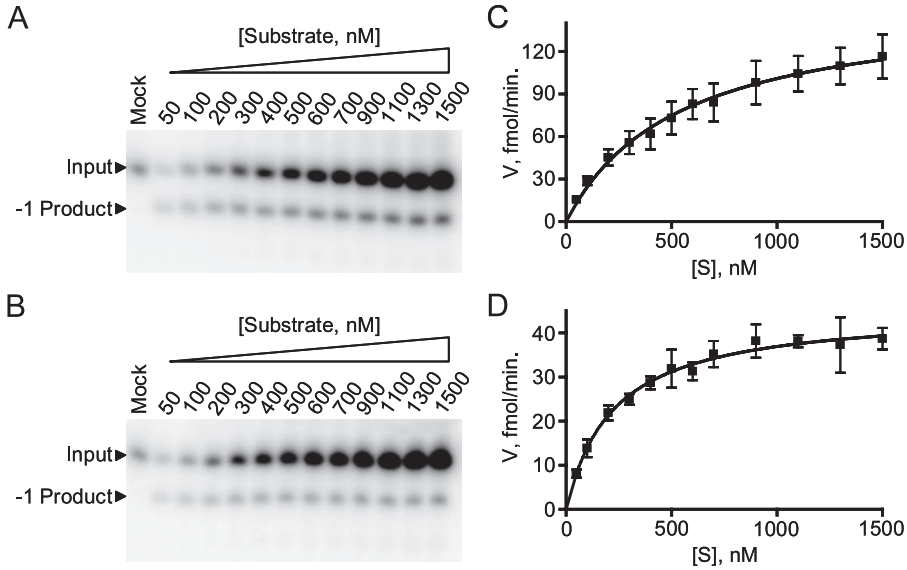

FIGURE 5. **Kinetic analysis of RNA hydrolysis by r*Tb*REX1 and r*Tb*REX2 enzymes.** REX enzymes were incubated with varying concentrations of 1U RNA substrate and reaction products separated by denaturing 8 M urea PAGE. *A,* representative reaction with r*Tb*REX1; *B,* representative reaction with r*Tb*REX2 at the indicated substrate concentrations. Mock reactions without enzyme were used to determine background hydrolysis. Mean initial velocities (*solid boxes*) from three experiments were plotted *versus* substrate concentration for r*Tb*REX1 (*C*) and r*Tb*REX2 (*D*). Best fit regression (*solid line*) and standard deviation (*error bars*) are indicated. K_m constants were derived from nonlinear regression Michaelis-Menten plots.

bination was competent for editing but the r*Lm*REX1 and r*Lm*REL2 combination was not (1).

DISCUSSION

We have demonstrated that *Tb*REX1 and *Tb*REX2 are single-stranded U-specific 3′–5′-exoribonucleases. This is consistent with our previous characterization of the *Leishmania* EEP motif protein, *Lm*REX1 (1), and with the known specificity of U-deletion exouridylase activity in mitochondrial lysates. Mutations of conserved residues in the *Tb*REX1 and *Tb*REX2 EEP domains greatly reduced exonuclease activity, indicating that these domains are required for exonuclease catalysis. This was confirmed by showing that a truncated *Tb*REX1 peptide containing only the EEP domain is sufficient for U-specific exonuclease activity. This is consistent with our finding that recombinant *Lm*REX2*, which lacks this motif, has no detectable exonuclease activity. Together, these results represent the first demonstrated difference in enzymatic content of the *Leishmania* and *Trypanosoma* L-complexes, raising the possibility that other differences may exist.

The kinetoplastid REX1 and REX2 proteins are the only known 3′–5′ U-specific exonucleases. No orthologues of these enzymes have been identified outside the order Kinetoplastida; however, other EEP motif proteins may provide some insight into REX function. Although nucleotide-specific exonuclease activities are rare, the EEP protein family members CCR4 and Nocturnin, two

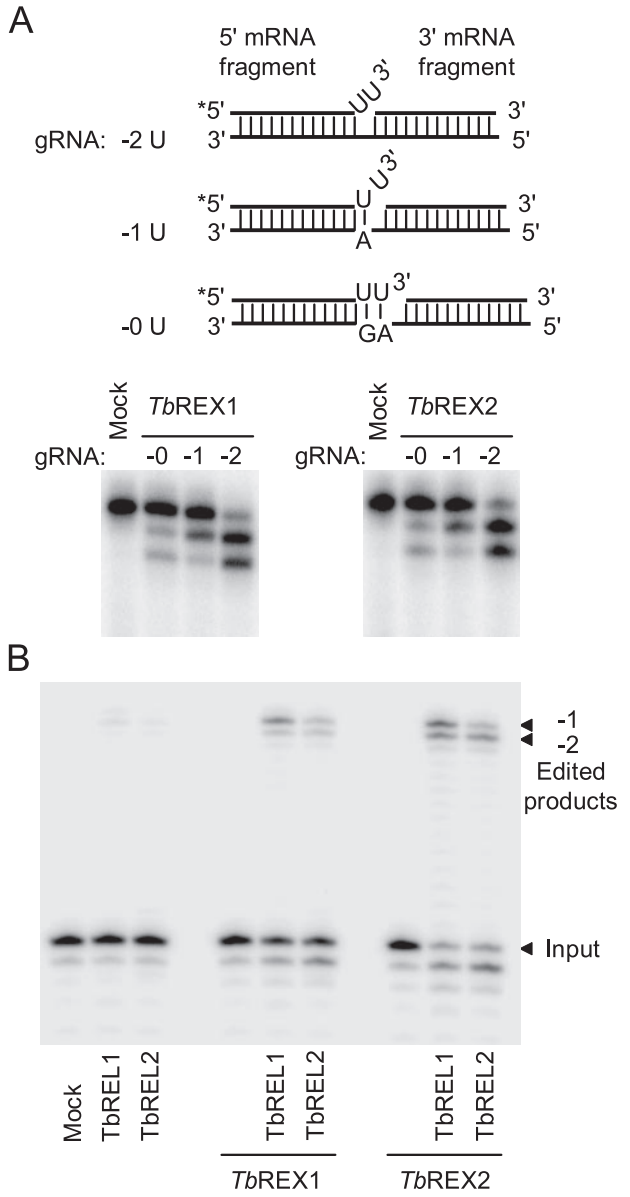

FIGURE 6. **r*Tb*REX1 and r*Tb*REX2 exonuclease activities are gRNA-directed.** *A,* REX proteins were incubated with pre-cleaved deletion editing substrates with different gRNA sequences as shown in the diagram. Base-pairing between guiding nucleotides of the gRNA and 3′ Us reduces *Tb*REX activity. *B,* reconstitution of pre-cleaved U deletion editing with r*Tb*REX and r*Tb*REL proteins. −1 U and −2 U fully edited products were generated from the −2U-deletion substrate with any combination of r*Tb*REX and r*Tb*REL proteins.

3′–5′ RNA deadenylases, may provide the closest functional models for the REX proteins (29–31). CCR4 exhibits both distributive and processive activity in a substrate-dependent fashion, possibly as a function of mRNA length (32). We showed that the highly purified *T. brucei* REX proteins exhibit a distributive activity, but proteins purified only by affinity chromatography show a processive activity (data not shown).

Homology modeling has suggested that REX EEP domains may adopt a four-layer α/β fold similar to that of EEP proteins with known structures (27), and consequently, *Tb*REX enzymes may utilize a catalytic mechanism similar to those described for other EEP proteins. However, although conserved residues that characterize the EEP motif are located in a single active site, distinct catalytic mechanisms have been proposed for various EEP model proteins (33, 34). Our double amino acid mutations of *Tb*REX1(D881A,H882N), and *Tb*REX2(D896A,H897N) were based on functional analyses of the EEP motif protein, APE1, an apurinic endonuclease involved in base excision repair. Single amino acid mutations of equivalent residues in APE1 have demonstrated that these residues are important for catalytic activity (35, 36), and crystal structures of APE1 have confirmed the localization of these residues at the active site (33, 37). Further mutational analysis may provide some insight into the exact catalytic mechanism of REX proteins.

A 5–10 S subcomplex containing *Tb*REL1, *Tb*MP63, and *Tb*REX2 has been identified by gradient sedimentation of *Tb*REL1 TAP-purified L-complex and found to be capable of pre-cleaved deletion editing (18). A similar subcomplex containing *Lt*REX2*, *Lt*REL1, and *Lt*LC4 has been identified in *L. tarentolae* (6). However, the lack of enzymatic activity of *Lm*REX2* suggests that that in *Leishmania* this subcomplex may not be involved in U-deletion editing. The role of *Leishmania* REX2* remains unclear. One possibility is that *Lm*REX2* may simply play a structural role, perhaps stabilizing interactions within the REL1 subcomplex. Presumably, REX1 acts as the primary U-deletion editing exonuclease in the *Leishmania* system. Interactions between REX1 and other L-complex components have yet to be identified. It remains possible that the *Leishmania* REX1 protein may functionally interact with the REL1 sub-complex *in vivo* or substitute for *Lt*REX2* in a portion of the total subcomplexes.

Analysis of the mRNA cleavage step of editing in gradient-purified 20 S fractions first indicated that separate endonucleases act at insertion and deletion editing sites (38). Endonuclease activity preferentially at U-deletion editing sites of the L-complex protein, *Tb*REN1, have been confirmed *in vitro* with recombinant REN1 (5) and *in vivo* through mutation of the *Tb*REN1 RNase III motif (3). A U-insertion site preference of another RNase III endonuclease, *Tb*REN2, has been identified *in vivo* (4). *Tb*REN1 and *Tb*REN2 appear to be mutually exclusive in L-complexes isolated by either *Tb*REN1 TAP or *Tb*REN2 TAP (39). However, although *Tb*REX2 was present in both L-complexes, *Tb*REX1 was only identified in the *Tb*REN1 complex (39).

The similarity of r*Tb*REX1 and r*Tb*REX2 activities *in vitro* suggests that these enzymes may act redundantly *in vivo* and may both play catalytic roles in U-deletion editing. This is supported by the successful reconstitution of pre-cleaved deletion editing with either *Tb*REX enzyme. The minimal phenotype of down-regulated *Tb*REX2 cells and the slow growth phenotype of down-regulated *Tb*REX1 cells (1) are consistent with a limited ability of REX enzymes to act redundantly *in vivo*. The stronger growth phenotype resulting from down-regulation of *Tb*REX1 (1) suggests that *Tb*REX2 may not fully substitute for *Tb*REX1 function. However, RNAi down-regulation of *Tb*REX1 expression produces a shift in sedimentation value of the L-complex greater than that observed upon *Tb*REX2 down-regulation. We cannot discount the possibility that the slow growth phenotype of down-regulated *Tb*REX1 cells is because of loss of other L-complex components rather than an inability of *Tb*REX2 to compensate for loss of *Tb*REX1. The REX pro-

teins might also have distinct *in vivo* functions in *T. brucei*, including specificity for specific deletion substrates or trimming of excess 3′ Us from the 5′ cleavage fragment possibly added by RET1 or RET2 terminal uridylyltransferase activity at U-deletion or U-insertion sites (40–42).

The presence of several homologues of editing proteins, one of which is required and the other nonessential, has been also demonstrated for the RNA ligases REL1 and REL2. It is not clear if this organizational trend in editing proteins represents a functional organization or is an evolutionary artifact.

Acknowledgments—We thank all members of the Simpson Laboratory for advice and discussion. We thank Ken Stuart (Seattle Biomedical Research Institute, Seattle) for the gift of monoclonal antibodies against TbLC1, TbLC4, TbREL1, and TbLC7b and Arthur Gunzl (University of Connecticut Health Center) for the gift of the protein C-containing TAP vector.

REFERENCES

1. Kang, X., Rogers, K., Gao, G., Falick, A. M., Zhou, S.-L., and Simpson, L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1017–1022
2. Blum, B., Bakalara, N., and Simpson, L. (1990) *Cell* **60,** 189–198
3. Trotter, J. R., Ernst, N. L., Carnes, J., Panicucci, B., and Stuart, K. (2005) *Mol. Cell* **20,** 403–412
4. Carnes, J., Trotter, J. R., Ernst, N. L., Steinberg, A., and Stuart, K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 16614–16619
5. Kang, X., Gao, G., Rogers, K., Falick, A. M., Zhou, S., and Simpson, L. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 13944–13949
6. Aphasizhev, R., Aphasizheva, I., Nelson, R. E., Gao, G., Simpson, A. M., Kang, X., Falick, A. M., Sbicego, S., and Simpson, L. (2003) *EMBO J.* **22,** 913–924
7. Stuart, K. D., Schnaufer, A., Ernst, N. L., and Panigrahi, A. K. (2005) *Trends Biochem. Sci.* **30,** 97–105
8. Simpson, L., Aphasizhev, R., Gao, G., and Kang, X. (2004) *RNA* (*N. Y.*) **10,** 159–170
9. Gao, G., Simpson, A. M., Kang, X., Rogers, K., Nebohacova, M., Li, F., and Simpson, L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 4712–4717
10. McManus, M. T., Shimamura, M., Grams, J., and Hajduk, S. L. (2001) *RNA* (*N. Y.*) **7,** 167–175
11. Rusche, L. N., Huang, C. E., Piller, K. J., Hemann, M., Wirtz, E., and Sollner-Webb, B. (2001) *Mol. Cell. Biol.* **21,** 979–989
12. Aphasizhev, R., Aphasizheva, I., and Simpson, L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10617–10622
13. Ernst, N. L., Panicucci, B., Igo, R. P., Jr., Panigrahi, A. K., Salavati, R., and Stuart, K. (2003) *Mol. Cell* **11,** 1525–1536
14. Stuart, K., Panigrahi, A. K., and Schnaufer, A. (2004) *Methods Mol. Biol.* **265,** 273–291
15. Panigrahi, A. K., Schnaufer, A., Ernst, N. L., Wang, B., Carmean, N., Salavati, R., and Stuart, K. (2003) *RNA* (*N. Y.*) **9,** 484–492
16. Panigrahi, A. K., Allen, T. E., Stuart, K., Haynes, P. A., and Gygi, S. P. (2003) *J. Am. Soc. Mass Spectrom.* **14,** 728–735
17. Stuart, K., Panigrahi, A. K., Schnaufer, A., Drozdz, M., Clayton, C., and Salavati, R. (2002) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357,** 71–79
18. Schnaufer, A., Ernst, N. L., Palazzo, S. S., O'Rear, J., Salavati, R., and Stuart, K. (2003) *Mol. Cell* **12,** 307–319
19. Schimanski, B., Nguyen, T. N., and Gunzl, A. (2005) *Eukaryot. Cell* **4,** 1942–1950
20. Coburn, C. M., Otteman, K. M., McNeely, T., Turco, S. J., and Beverley, S. M. (1991) *Mol. Biochem. Parasitol.* **46,** 169–179
21. Hutchings, N. R., Donelson, J. E., and Hill, K. L. (2002) *J. Cell Biol.* **156,** 867–877
22. Wickstead, B., Ersfeld, K., and Gull, K. (2002) *Mol. Biochem. Parasitol.* **125,** 211–216
23. Carruthers, V. B., and Cross, G. A. M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8818–8821
24. Braly, P., Simpson, L., and Kretzer, F. (1974) *J. Protozool.* **21,** 782–790
25. Dlakic, M. (2000) *Trends Biochem. Sci.* **25,** 272–273
26. Worthey, E. A., Schnaufer, A., Mian, I. S., Stuart, K., and Salavati, R. (2003) *Nucleic Acids Res.* **31,** 6392–6408
27. Mian, I. S., Worthey, E. A., and Salavati, R. (2006) *BMC Bioinformatics* **7,** 305
28. Igo, R. P., Jr., Weston, D. S., Ernst, N. L., Panigrahi, A. K., Salavati, R., and Stuart, K. (2002) *Eukaryot. Cell* **1,** 112–118
29. Tucker, M., Staples, R. R., Valencia-Sanchez, M. A., Muhlrad, D., and Parker, R. (2002) *EMBO J.* **21,** 1427–1436
30. Chen, J., Chiang, Y. C., and Denis, C. L. (2002) *EMBO J.* **21,** 1414–1426
31. Baggs, J. E., and Green, C. B. (2003) *Curr. Biol.* **13,** 189–198
32. Viswanathan, P., Chen, J., Chiang, Y. C., and Denis, C. L. (2003) *J. Biol. Chem.* **278,** 14949–14955
33. Mol, C. D., Izumi, T., Mitra, S., and Tainer, J. A. (2000) *Nature* **403,** 451–456
34. Mol, C. D., Kuo, C. F., Thayer, M. M., Cunningham, R. P., and Tainer, J. A. (1995) *Nature* **374,** 381–386
35. Barzilay, G., Walker, L. J., Robson, C. N., and Hickson, I. D. (1995) *Nucleic Acids Res.* **23,** 1544–1550
36. Chou, K. M., and Cheng, Y. C. (2003) *J. Biol. Chem.* **278,** 18289–18296
37. Beernink, P. T., Segelke, B. W., Hadi, M. Z., Erzberger, J. P., Wilson, D. M., III, and Rupp, B. (2001) *J. Mol. Biol.* **307,** 1023–1034
38. Cruz-Reyes, J., Rusche, L., Piller, K. J., and Sollner-Webb, B. (1998) *Mol. Cell* **1,** 401–409
39. Panigrahi, A. K., Ernst, N. L., Domingo, G. J., Fleck, M., Salavati, R., and Stuart, K. D. (2006) *RNA* (*N. Y.*) **12,** 1038–1049
40. Cruz-Reyes, J., and Sollner-Webb, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8901–8906
41. Byrne, E. M., Connell, G. J., and Simpson, L. (1996) *EMBO J.* **15,** 6758–6765
42. Zhelonkina, A. G., O'Hearn, S. F., Law, J. A., Cruz-Reyes, J., Huang, C. E., Alatortsev, V. S., and Sollner-Webb, B. (2006) *RNA* (*N. Y.*) **12,** 476–487