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# Role of Uridylate-Specific Exoribonuclease Activity in *Trypanosoma brucei* RNA Editing

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**Editing of mitochondrial mRNAs in kinetoplastid protozoa occurs by a series of enzymatic steps that insert and delete uridylates (U's) as specified by guide RNAs (gRNAs). The characteristics of the 3′ exonuclease activity that removes the U's following cleavage during deletion editing were determined by using an in vitro precleaved deletion assay that is based on ATPase subunit 6 pre-mRNA and gA6[14] gRNA. The exonuclease in partially purified editing complexes is specific for U's. The specificity occurs in the absence of gRNA, but its activity is enhanced by the presence of gRNA. The 3′ pre-mRNA fragment enhances the specificity, but not the efficiency, of U removal. The activity is sensitive to the 5′ phosphate of the 3′ fragment, which is not required for U removal. The ability of the 3′ U's to base pair with purines in the gRNA protects them from removal, suggesting that the U-specific 3′ exonuclease (exoUase) is specific for U's which are not base paired. ExoUase is stereospecific and cannot remove ($R_p$)α-thio-U. The specificity of the exoUase activity thus contributes to the precision of RNA editing.**

RNA editing is an unusual mRNA maturation process which occurs in the mitochondria of kinetoplastids, a group of flagellate protozoans that includes *Trypanosoma brucei*, the organism responsible for African sleeping sickness. RNA editing employs a series of enzymatic steps to insert and delete uridylates (U's) in mitochondrial (mt) mRNAs as specified by guide RNAs (gRNAs) (for reviews, see references 12, 18, and 37). The steps begin with an endonucleolytic cleavage of the pre-mRNA at the editing site (ES), which is generally immediately 5′ of a duplex formed between the gRNA and pre-mRNA. In deletion editing, U's are removed from the 3′ terminus of the 5′ cleavage fragment by a U-specific 3′ exonuclease (exoUase) and are released as free UMP (31). In insertion editing, U's are added to the 3′ end by terminal uridylyl transferase (TUTase) activity. Finally, the processed 5′ fragment is rejoined to the 3′ fragment by RNA ligase, concluding one round of RNA editing. The number of U's inserted or deleted is determined by the sequence of the informational region of gRNA, which is complementary to edited RNA by Watson-Crick and G · U base pairing (4).

RNA editing is catalyzed by a large multiprotein complex. This complex contains all of the enzymatic activities required for editing sediments at 20S on glycerol gradients (6, 8, 30, 31), and the characterization of the components of the editing complex is under way (20, 23, 28, 29; M. Drożdż, R. Salavati, J. O'Rear, S. S. Palazzo, R. P. Igo, Jr., C. Clayton, and K. Stuart, unpublished data). Of the enzymes required for the catalytic steps, only RNA ligase has been unequivocally identified (25, 32, 34). Two RNA ligases have been identified in the editing complex, but only one, TbMP52, is required for editing in *T. brucei* (32, 34). An RNA helicase, mHel61p, also appears to function in editing, perhaps by unwinding gRNA–pre-mRNA duplexes (27).

Little is known about the editing 3′ exoUase. A candidate editing 3′ exoUase activity has been partially purified (2), but the gene has not yet been identified. Recent studies of the editing 3′ exoUase, in which an in vitro editing assay that requires endonucleolytic cleavage was used, suggest that the activity is specific for U's (9, 22a, 35, 36). ExoUase activity may play several roles associated with RNA editing. These include the removal of U's from 5′ pre-mRNA cleavage fragments during deletion editing (4), the removal of excess U's that may be added during insertion editing (5), and the removal of posttranscriptionally added U's from the 3′ tails of gRNAs (24). We report here the results of a study of deletion editing focused on the editing 3′ exoUase activity. In this study, we used partially purified editing complexes and a precleaved-mRNA in vitro assay in which the substrates mimic the A6 pre-mRNA fragments resulting from cleavage at ES1 (3, 35, 36). This allowed the study of the U-removal and RNA ligation steps of deletion editing without endonucleolytic cleavage.

## MATERIALS AND METHODS

**Cell culture and preparation of mt extract.** The growth of *T. brucei brucei* strain IsTaR 1.7a cells and the purification of mitochondria by centrifugation in Percoll gradients were carried out as described previously (15, 28). mt extract was purified either by sequential SP Sepharose and Q Sepharose ion-exchange chromatography (all experiments except those shown in Fig. 1) or by these two steps plus Superose-6 gel filtration chromatography (28). The final KCl concentration in both types of extract was 200 mM.

**Preparation of RNAs.** Unless otherwise stated, all RNAs were prepared by T7 transcription from PCR templates. The PCR template for the 5′ precleaved RNA (U5-5′CL) was prepared by annealing the U5-5′ antisense oligonucleotide (5′-AAAATCACAACTTTCCCTTTCCTATAGTGAGTCGTATTAC-3′) with the EcoRIT7 sense oligonucleotide (5′-CGGCGGAATTCTGTAATACGACT CAC-3′). "Overhangs" were filled in by the Klenow enzyme, and PCR was performed with EcoRIT7 and the antisense primer U5-5′short (5′-AAAATCA CAACTTTCCCT-3′). All mutant precleaved A6U5-5′ RNAs were prepared as

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described above but with their respective antisense primers: U5-5′CL1A RNA with template primer U5-5′A1 (5′-TAAATCACAACTTTCCCTTTCCTATAG TGAGTCGTATTAC-3′) and PCR primer U5-5′A1short (5′-TAAATCACAA CTTTCC-3′); U5-5′CL2A RNA with template primer U5-5′A2 (5′-ATAATCA CAACTTTCCCTTTCCTATAGTGAGTCGTATTAC-3′) and PCR primer U5-5′A2short (5′-ATAATCACAACTTTCC-3′); U5-5′CL3A RNA with template primer U5-5′A3 (5′-AATATCACAACTTTCCCTTTCCTATAGTGAGTCGT ATTAC-3′) and PCR primer U5-5′A3short (5′-AATATCACAACTTTCC-3′); U5-5′CL4A RNA with template primer U5-5′A4 (5′-AAATTCACAACTTTC CCTTTCCTATAGTGAGTCGTATTAC-3′) and PCR primer U5-5′A4short (5′-AAATTCACAACTTTCC-3′); and 5′CL18 RNA with template primer 5′CL18-Tmp1 (5′-GGCGGAATTCTGTAATACGACTCACTATAG-3′) and PCR primer 5′CL18-3′ (5′-CGGCGGAATTCTGTAATACGACTCACTATA G-3′). The 3′ precleaved A6U5-3′ RNAs U5-3′CLpp and U5-3′CL 3′p, with and without a 5′ phosphate, respectively, were synthesized by Oligos, Etc. The complementary gA6[14]PC-del gRNA PCR template was prepared by annealing the A6comp1 antisense oligonucleotide (5′-GGAAAGGGAAAGTTGTGAGCGAGT TATAGAACCTATAGAACCTATAGTGAGTCGTATTAC-3′) with EcoRIT7 and extended with the Klenow enzyme. All mutant gRNA templates were prepared as described above but with their respective antisense templates: gA6comp2 RNA with A6comp2 (5′-GGAAAGGGAAAGTTGTGTGCGAGTTATAGAACCTAT AGAACCTATAGTGAGTCGTATTAC-3′), gA6comp3 RNA with A6comp3 (5′ - GGAAAGGGAAAGTTGTGCGCGAGTTATAGAACCTATAGAACCT ATAGTGAGTCGTATTAC-3′), gA6comp4 RNA with A6comp4 (5′-GGAAAG GGAAAGTTGTGGGCGAGTTATAGAACCTATAGAACCTATAGTGAGTC GTATTAC-3′), gA6comp1-0 RNA with A6comp1-0 (5′-GGAAAGGGAAAGTT GTGATTTTGCGAGTTATAGAACCTATAGAACCTATAGTGAGTCGTATT AC-3′), gA6comp1-1 RNA with A6comp1-1 (5′-GGAAAGGGAAAGTTGTGAT TTGCGAGTTATAGAACCTATAGAACCTATAGTGAGTCGTATTAC - 3′), gA6comp1-2 RNA with A6comp1-2 (5′-GGAAAGGGAAAGTTGTGATTGCGA GTTATAGAACCTATAGAACCTATAGTGAGTCGTATTAC-3′), gA6comp1-3 RNA with A6comp1-3 (5′-GGAAAGGGAAAGTTGTGATGCGAGTTATAGA ACCTATAGAACCTATAGTGAGTCGTATTAC-3′), gA6[14]PCdel-AG RNA with oligo(A6compAG) (5′-GGAAAGGGAAAGTTGTGATCGCGAGTTATAG AACCTATAGTGAGTCGTATT-3′), gA6[14]PCdel-AC with oligo(A6compAC) (5′ - GGAAAGGGAAAGTTGTGATGGCGAGTTATAGAACCTATAGTGA GTCGTATT-3′), gA6[14]PCdel-GG with oligo(A6compGG) (5′-GGAAAG GGAAAGTTGTGACCGCGAGTTATAGAACCTATAGTGAGTCGTATT-3′), gA6[14]PCdel-GC with oligo(A6compGC) (5′-GGAAAGGGAAAGTTGTGA CGGCGAGTTATAGAACCTATAGTGAGTCGTATT-3′), and gA6[14]PCdel-CC with oligo(A6compCC) (5′-GGAAAGGGAAAGTTGTGAGGGCGAGTTA TAGAACCTATAGTGAGTCGTATT-3′). All gRNA templates were amplified by PCR with the antisense primer ASgRNA (5′-GGAAAGGGAAAGTTGTG-3′) and EcoRIT7. About 5 μg of each PCR template was used to produce in vitro transcripts with T7 RNA polymerase (Promega). The 5′ precleaved RNAs were alkaline phosphatase treated and labeled by phosphorylation by using T4 polynucleotide kinase (Gibco-BRL) in the presense of [γ-$^{32}$P]ATP (NEN). All RNAs were gel purified by 15 or 20% (wt/vol) denaturing (7 M urea) polyacrylamide gel electrophoresis.

Since T7 RNA polymerase is specific for ($S_P$)α-thio-nucleotide triphosphates and inverts the $S_P$ phosphorothioate linkage to $R_P$ during transcription (14), U5-5′CL containing ($R_P$)α-thio-U residues was prepared by T7 transcription (Promega) in a reaction mixture containing 4.5 mM ($S_P$)α-thio-UTP (NEN) and 5 mM (each) ATP, CTP, and UTP. Transcription products were gel purified by 20% (wt/vol) denaturing (7 M urea) polyacrylamide gel electrophoresis. The *T. brucei* mt TUTase was used to generate 5′CL18 RNA with 1 and 2 3′-terminal ($S_P$)α-thio-U residues by following the procedure and using the mt extract developed by N. L. Ernst (unpublished data). The identities of these linkages were assessed by digesting RNA with nucleases specific for either the $R_P$ or the $S_P$ diastereomer (13, 14; Ernst, unpublished).

**U removal and editing assays.** Exonuclease and deletion editing assays using the precleaved deletion substrate (Fig. 1A) were adapted from in vitro editing assays in use in the laboratory (19, 35, 38). Reaction mixtures generally contained 50 fmol of radiolabeled U5-5′CL, 1 pmol of U5-3′CL, and 0.5 pmol of gA6[14]PC-del combined with either 7 μl of SP Sepharose-Q Sepharose-purified mt extract (see Fig. 2 through 5) or 3 μl of SP Sepharose–Q Sepharose–Superose-6-purified mt extract (see Fig. 1) in a total volume of 30 μl in HHE buffer (25 mM HEPES [pH 7.9], 10 mM Mg diacetate, 5 mM $CaCl_2$, 50 mM KCl, 0.5 mM dithiothreitol). The reaction mixtures were then incubated for 3 h or longer at 28°C before the reactions were stopped by the addition of 2 μl of 260 mM EDTA–2.5% sodium dodecyl sulfate. Experiments using 3 μl of extract were designed to measure initial rates of U removal and ligation, and these reaction mixtures were incubated for 3 h. Modifications of this procedure are described in the text. Reaction mixtures were phenol-choroform extracted, and the RNA was precipitated; 10 pmol of nonradioactive U5-5′CL RNA was added to reduce the level of interaction of labeled RNA products with gRNA during electrophoresis. Before being loaded onto 18% (wt/vol) denaturing polyacrylamide gels, samples were heated at 100°C for 2 min. Labeled RNAs were visualized by using a Storm PhosphorImager (Molecular Dynamics). Reaction mixtures lacking mt extract were used as background controls for the quantification of editing products with ImageQuaNT software.

## RESULTS

**U removal and deletion editing of precleaved substrates.** A precleaved deletion editing assay was adapted from the precleaved insertion assay (17) by redesigning the 5′ and 3′ pre-mRNA substrates and gRNA. The pre-mRNA substrate fragments were based on the A6U5 substrate that has been used for in vitro deletion (35) (Fig. 1A). The 5′ fragment, U5-5′CL, has a sequence identical to that of a portion of A6U5 RNA immediately upstream of ES1. The sequence of the 3′ fragment, U5-3′CL, is identical to that of A6U5 immediately downstream of ES1, except that two nucleotides at the 5′ end were changed to provide stronger base pairing with the gRNA. The gA6[14]PC-del gRNA is complementary to the U5-5′CL and U5-3′CL fragments and specifies the deletion of four U's. Incubation of the substrate RNAs, gRNA, and substantially purified editing complexes (28), as described in Materials and Methods, resulted in the removal of four U's (i.e., removal to the first non-U nucleotide) and preferential ligation with the 3′ fragment of the 5′ fragment from which all four U's had been removed (Fig. 1B, lane 5). U's were removed from the 3′ end of U5-5′CL in the absence of the gRNA (lanes 2 and 3), but the removal of all four U's was enhanced 10- to 20-fold in the presence of gRNA (lanes 4 through 6). Omission of the 3′ fragment did not affect the efficiency of U removal but did increase the relative abundance of the −3 removal product compared to that of the −4 product (compare lanes 4 and 5). This, along with the low level of U removal from the 5′ fragment alone (lane 2), suggests that there is little nonspecific exonuclease activity in the editing complexes which were purified by sequential SP Sepharose, Q Sepharose, and Superose-6 chromatography (28) (Fig. 1B). It was difficult to estimate the fold purification of the U-removal activity, because the level of activity in crude mt lysates was very low, perhaps due to endogenous protease activity, and because crude lysates contained abundant non-nucleotide-specific 3′ exonuclease activity, which digested input RNA (data not shown).

A low level of ligation activity was found in the absence of added ATP due to the presence of adenylylated RNA ligase (Fig. 1B, lane 5) (17, 33). The addition of 300 μM ATP increased, by over eightfold, ligation of the −4 U-removal product with U5-3′CLpp, resulting in accurately edited RNA (lane 6). ATP at 300 μM was saturating for ligation (data not shown). ATP also promoted ligation of 5′ fragments with fewer than four U's removed, especially of the input RNA. Thus, the addition of ATP promotes ligation of splinted RNAs that have an overhang as well as RNAs with a gap (17; S. S. Palazzo, A. K. Panigrahi, R. P. Igo, Jr., and K. Stuart, unpublished data). Nevertheless, accurate editing is largely dependent on ATP (lane 6 and data not shown), unlike the precleaved insertion editing catalyzed by the same mt extract (reference 17 and data not shown). The presence of ATP caused a one-third decrease in the total accumulation of −4 removal fragments (nonligated

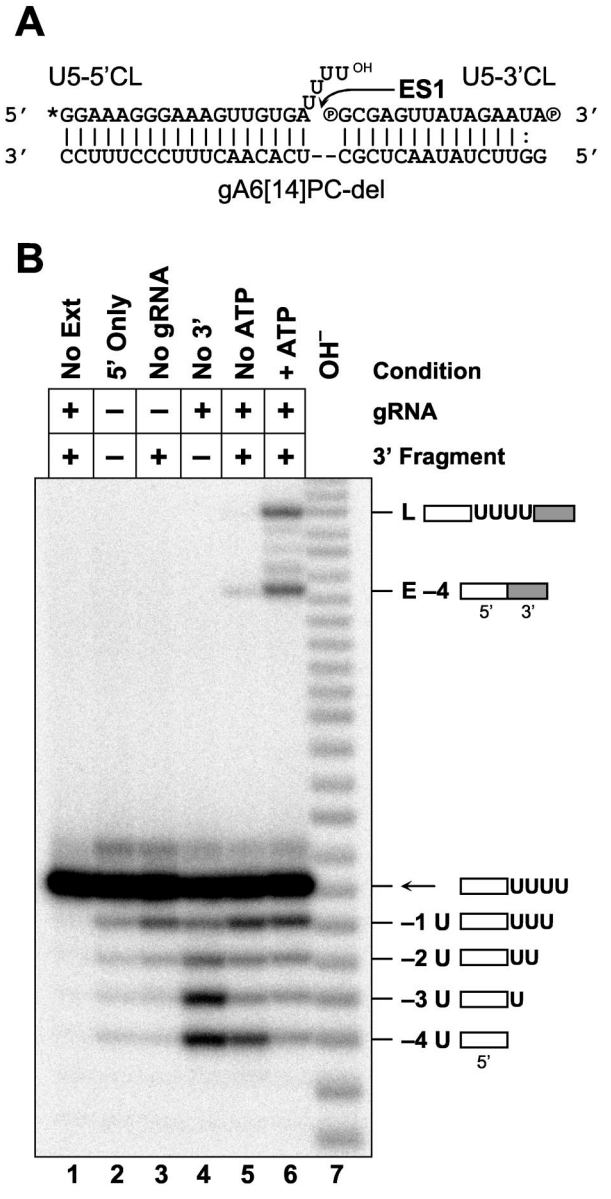


FIG. 1. Accurate deletion editing of the precleaved substrate. (A) Diagram of the precleaved deletion substrate annealed to gRNA. The four overhanging U's to be removed extend above the RNA-RNA duplex. The 3′ fragment contains 5′ and 3′ monophosphates, as shown. An asterisk indicates the [$^{32}$P]phosphate radiolabel. Hyphens have been added in gA6[14]PC-del at the ES (ES1) for clarity; the RNA strand is continuous across the ES. (B) Autoradiogram of RNA products of the precleaved deletion reaction. Reaction mixtures lacking one or more of the RNAs in panel A are labeled as follows: 5′ Only, U5-5′CL only; No gRNA, U5-5′CL and 3′CL in a 1:20 molar ratio; and No 3′, U5-5′CL and gA6[14]PC-del in a 1:2 molar ratio. The +ATP lane contained 300 μM ATP. All reaction mixtures except those lacking mt extract (No Ext) contained U5-5′CL, 3′CLpp, and gA6[14]PC-del in a 1:20:10 molar ratio and 3 μl of *T. brucei* mt extract. For clarity, the presence (+) or absence (−) of gA6[14]PC-del (gRNA) and U5-3′CLpp (3′ Fragment) in each lane is indicated. The input U5-5′CL RNA is indicated with an arrow. 5′ fragments with one, two, three, or four 3′-terminal U's removed are labeled −1 U through −4 U, respectively; L, ligation product of U5-5′CL with no missing U's and with U5-3′CL; E −4, edited RNA with four U's deleted. Diagrams of the editing products are also provided; the 5′ and 3′ fragments are represented by open and shaded rectangles, respectively, and the U's remaining at the 3′ terminus of U5-5′CL are shown. An alkaline hydrolysis ladder ($OH^-$) is included as a size standard.

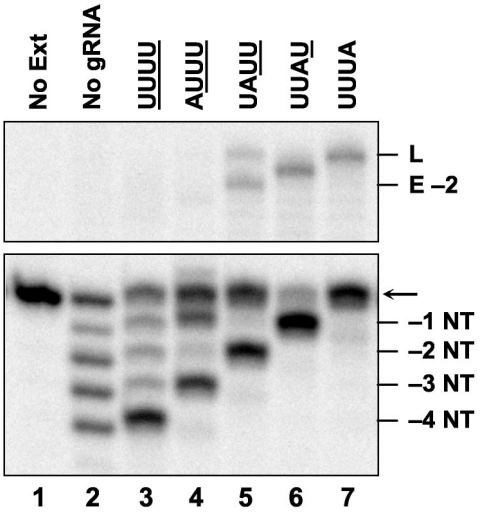


FIG. 2. U specificity of *T. brucei* mt exonuclease. The 3′ end sequence of the 5′ fragment included in each reaction is shown. U's exposed at the 3′ terminus are underlined. Wild-type U5-5′CL was used in reaction mixtures without mt extract (No Ext) and without gRNA. Products marked −1 NT through −4 NT have 1 through 4 nucleotides removed from the 3′ end, respectively; 5′ fragments that have no (L) or 2 (E −2) nucleotides removed and that are ligated to 3′ fragments are indicated.

plus ligated), possibly because the RNA ligase activity competes with that of exoUase for the available input 5′ fragment (Fig. 1B, compare lanes 5 and 6).

The specificity of the mt exonuclease activity for U's was tested by assaying the removal of nucleotides from a 5′ fragment in which one of the four 3′-terminal U's was replaced by an A (Fig. 2). In each case, U's were efficiently removed from the 3′ end up to the substituted A, which was not removed, indicating a high specificity of the exoUase for U. The single U was removed very efficiently from the substrate with only one 3′-terminal U (Fig. 2, lane 6). Ligation products with zero, one, or two removed U's were generated from substrates with two or fewer 3′ U's (lanes 5 through 7), reflecting the abundance of these U-removal products. Enhanced ligation of 5′ fragments with an A near the 3′ end may have resulted from base pairing of the A with the U in gRNA upstream and adjacent to the ES (with respect to mRNA) and from subsequent alignment of the 3′ terminus for ligation. Similar results were obtained in the full-round deletion editing assay, when each U to be removed at ES1 in intact A6U5 was replaced by a C (22a).

The kinetics of U removal from a 5′ fragment bound to gRNA was examined (Fig. 3). In the presence of 300 μM ATP, the nonligated −4 removal product accumulated quickly during the first hour, leveled off, and increased much more slowly over the next 6 h (Fig. 3A and B). In contrast, the accurately edited RNA product accumulated steadily over the 8-h time course, although it did so somewhat less quickly during the last 4 h. The number of ligated 5′ fragments with no U's removed also increased steadily, but to a lower level than that of the edited RNA (Fig. 3A). The 5′ phosphate of the 3′ fragment was necessary for efficient U removal during precleaved-mRNA deletion reactions without ATP (to prevent ligation) (Fig. 3C). The initial rate of accumulation of the −4 removal product was more than 100-fold higher with the 5′ phosphate

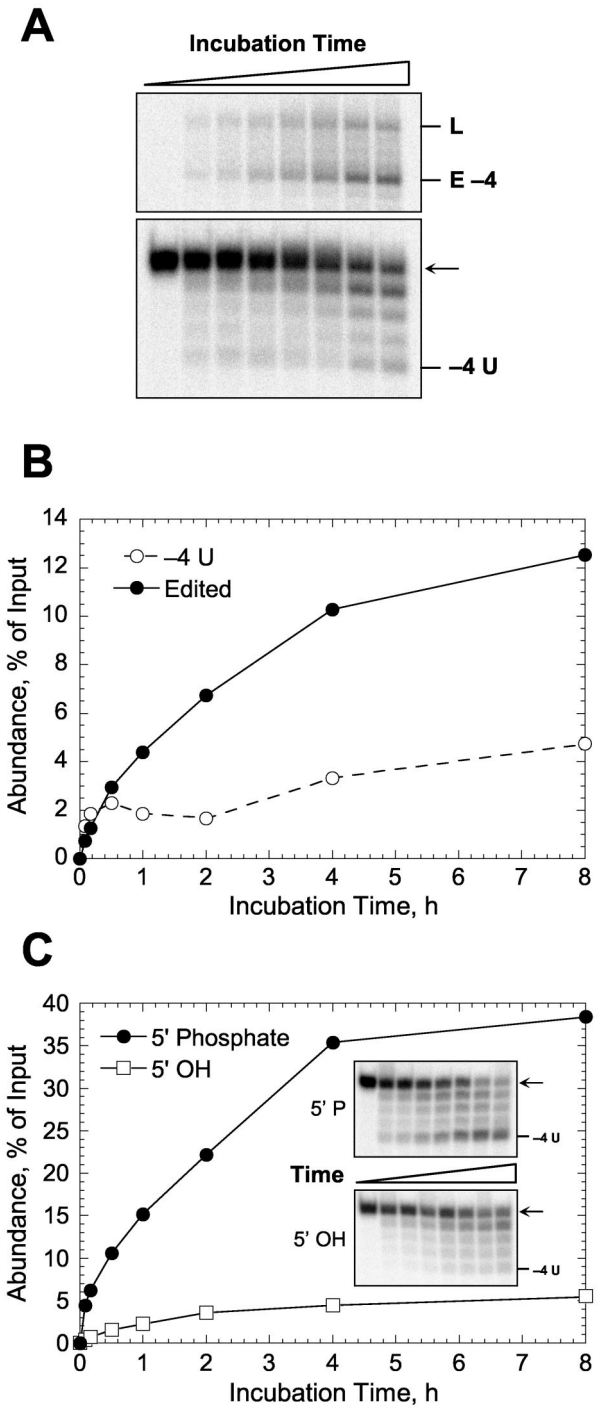


FIG. 3. Time course of U removal and precleaved deletion editing. (A) Time course of precleaved deletion editing in the presence of ATP. Reaction mixtures were incubated at 28°C for periods of time between 0 and 8 h. RNA species are designated as described for Fig. 1B. (B) Accumulation of U-removal products. The −4 U nonligated product and the −4 edited product in panel A were quantified as percentages of the total input RNA. (C) U removal without ATP. Reaction mixtures included a 3′ fragment with a 5′ phosphate or without a 5′ phosphate (5′ OH). Reaction mixtures were incubated for lengths of time between 5 min and 8 h, and the abundance of the −4 U-removal product was measured relative to the level of total input RNA. The same data are shown in the gel insets, with the input RNA (arrow) and −4 removal product indicated. Ligated products, which always totaled less than 1% of input, are not shown in the gel insets.

present than without, and although U removal gradually slowed after the first 5 min, after 8 h the −4 product was still more than seven times as abundant in the presence of the 5′ phosphate. These results were unexpected, given the high level of U removal in the absence of the 3′ fragment (Fig. 1B). They imply that the exoUase activity detected here is sensitive to the context of the 3′ terminus of the 5′ cleavage fragment, as also appears to be the case with U addition during precleaved insertion editing (17). U removal in the presence of a 3′ fragment with 5′ OH was dis- tributive (Fig. 3C, lower gel inset). The major product was −1 U even after 8 h, suggesting an alteration in exoUase activity. The initial U removal with the 5′ phosphate present was too rapid to determine whether it was distributive or processive (Fig. 3C, upper gel inset), but the lower relative abundance of removal products with fewer than four U's removed suggests that it is distributive (Fig. 3A and C, upper gel inset). This resembles U removal from a single, unpaired RNA by *Leishmania tarentolae* mt exoUase (2). Overall, the precleaved deletion is similar to full-round in vitro deletion (22a, 35) with only U's being removed from the 5′ fragment and their removal occurring quickly, while ligation proceeds more slowly.

**Protection of U's from removal by base pairing.** It has been proposed that guiding A's and G's protect U's that are added during insertion editing from removal by exoUase activity (5, 17), and such protection has been observed in the case of a completely double-stranded RNA with a 3′ U overhang (2). We tested this hypothesis using an RNA structure resembling that of an editing intermediate. U removal was examined when it was directed by variants of gA6[14]PC-del in which guiding nucleotides were inserted across from ES1 (Fig. 4). The presence of guiding A's, which can base pair with the 3′-terminal U's of U5-5′CL, resulted in considerable, but not complete, protection from removal by the exoUase (Fig. 4A). In the presence of 300 μM ATP, U's that would not base pair with guiding A's were removed, and these U-removal products were efficiently ligated to the 3′ fragment, as was the 5′ fragment with no U's removed (Fig. 4A, +ATP lanes). Very little removal of U's that could pair with the guiding A's was observed. In the absence of added ATP, the major U-removal product contained the same number of U's as there were guiding A's (Fig. 4A, −ATP lanes). Ligation was quite inefficient but reflected the major U-deletion product and thus resulted in accurately edited RNA (i.e., with the number of U's specified by the number of guiding A's). The incomplete protection from the removal of U's that can base pair with the guiding A's suggests out-of-step base pairing within the duplexes as well as breathing at the ends of the duplexes. Protection from removal depended on the ability of the 3′ U's to base pair with the guiding nucleotides (Fig. 4B). Two guiding C's (Fig. 4B, lane 1) did not block U removal past two U's; two guiding G's (lane 2) partially slowed removal of the third and fourth U's, while guiding AG (with A opposite the most 5′ U) (lane 4) protected U's from removal about as well as two guiding A's (lane 5). More removal beyond two U's was seen with guiding AC (lane 3) than with guiding GG. Thus, preferential accumulation of the −2 removal product was greatest when the two most 5′ U's could form base pairs with gRNA. Thus, base pairing of the guiding nucleotides, and not just their presence, prevents U removal. However, more −4 removal products accumulated

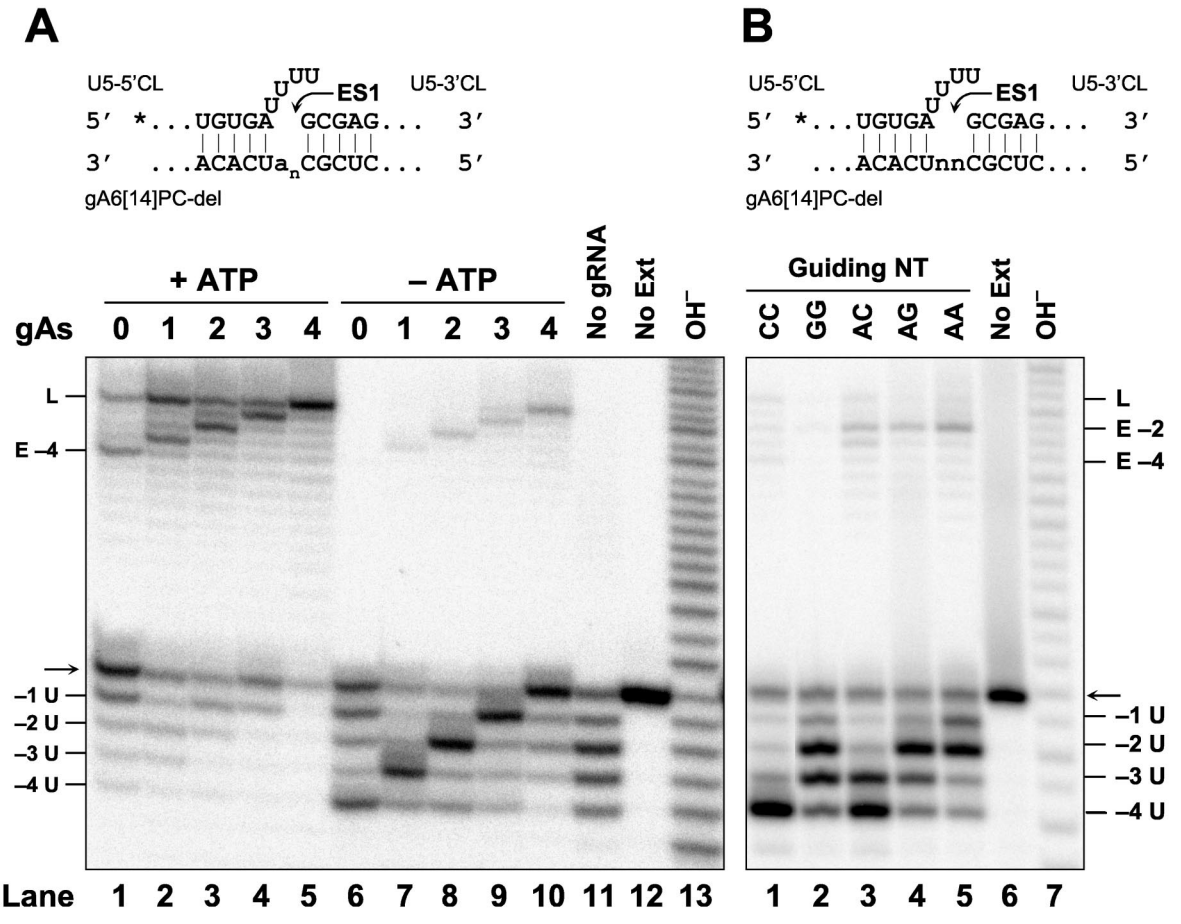


FIG. 4. Protection of 3′-terminal U's from removal. Control lanes and RNA products are labeled as described for Fig. 1. (A) Products from precleaved deletion reaction mixtures containing gRNA into which zero to four guiding A's (gAs; numbers of which are indicated by the numbers above the gel) were inserted at ES1. Reactions were performed in the presence (+ATP) or absence (−ATP) of 0.3 mM ATP. (B) Protection by various guiding nucleotides. The top panel shows the locations of guiding nucleotides (lowercase letters). gA6[14]PC-del used in these reactions contained two guiding nucleotides, as indicated above each lane, reading 3′ to 5′ with respect to gRNA (AA in no-extract lane).

with guiding AC than with a single guiding A (Fig. 4A, lane 7). This suggests that the sequence of gRNA affects 3′ exoUase activity in a more complex manner than simply through base pairing. Nevertheless, while U removal did not completely stop at −2 with guiding AC, the −2 edited product was the predominant ligated RNA for guiding AG and AA (Fig. 4B). Hence, ligation in this context was determined more by the number of guiding nucleotides than by their identities.

**Stereospecificity of U removal.** The stereospecificity of U removal by exoUase was examined by testing the susceptibilities of phosphorothioate analogs of U to the exoUase (Fig. 5). Exo Uase was unable to remove U's with 5′ $R_P$ phosphorothioate linkages (Fig. 5). U5-5′CL with four 3′-terminal ($R_P$)α-thio-U's was incubated with U5-3′CL and gA6[14]PC-del in a precleaved deletion assay. Essentially no $R_P$ linkages were cleaved by exoUase, and ligation of only the input RNA was observed. We also assessed the removal by exoUase of ($S_P$)α-thio-U by appending ($S_P$)α-thio-U to 5′CL18 RNA (17) by *T. brucei* TUTase in the presence of ($R_P$)α-thio-UTP, which was expected to convert it to the ($S_P$) diastereomer 13; N. L. Ernst, unpublished results). The incorporated U's were removed when this RNA was annealed with 3′13CLpp and gPCA6[14] (17) and incubated with purified editing complexes (data not shown). However, we cannot exclude the possibility that the removed U's were derived from traces of UTP in the ($R_P$)α-thio-UTP stock. Nevertheless, the exoUase stereospecificity is clearly against the $R_P$ diastereomer.

**Competition between U addition and U removal.** The ability of UTP to reduce the accumulation of U-removal products was examined to assess the relative rates of U addition and removal at the 3′ terminus of the 5′ cleavage fragment. UTP reduced the accumulation of the −4 removal product over a concentration range of several orders of magnitude, with a reduction of about 25% occurring at 1 mM (data not shown), a saturating concentration for U addition by the mt extract (Ernst et al., unpublished). The reduction is UTP specific, since the same concentrations of CTP did not significantly reduce accumulation of the −4 removal product. The reversal at 5 mM UTP or CTP may reflect nonspecific factors, such as binding of free nucleotides to the exoUase or to the editing complex or a change in pH. Similar results were obtained in the absence of gRNA (data not shown). UTP concentrations of up to 1 mM failed to prevent the low level of removal of U's that occurred when a gRNA with two A's was used to protect against the removal of two U's from U5-5′CL

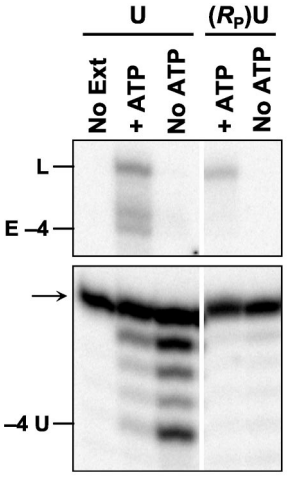


FIG. 5. Stereospecificity of mt exoUase. Conditions, U-removal products, and ligation products are labeled as described for Fig. 1B. Reactions were carried out in the presence (+ATP) or absence (−ATP) of 300 μM ATP. U5-5′CL containing standard U residues (U) and ($R_P$)α-thio-U's incorporated by T7 polymerase from ($S_P$)α-thio-UTP [($R_P$)U] was subjected to the precleaved-RNA deletion assay with U5-3′CL and gA6[14]PC-del.

(Fig. 4A, lane 8, and data not shown). Thus, U removal appears to be catalyzed more rapidly than U addition is catalyzed by the purified complexes, which may reflect a proofreading function of exoUase in its removal of excess U's added by TUTase during insertion editing.

## DISCUSSION

This study reports the characterization of the exonuclease activity that is associated with the editing complex by a precleaved deletion editing assay. The exonuclease activity is distributive and U specific, does not cleave $R_P$ diastereomers, and shows a preference for overhanging (i.e., non-base-paired) 3′ U's in a duplexed RNA molecule. The low level of inhibition of U-removal activity by UTP suggests that the exoUase may provide a form of proofreading by removing excess U's that are added by TUTase but are not specified by the gRNA. However, this possibility needs to be established more firmly through identification of the 3′ exoUase enzyme and characterization of its role in insertion editing, perhaps through gene inactivation studies.

The editing exonuclease activity has some characteristics that are similar to those of TUTase activity as well as some differences beyond the fact that the former removes U's while the latter adds them during editing. Both are specific for U's and are affected by the presence of gRNA and the presence of a 3′ pre-mRNA fragment, especially its 5′ phosphate (17, 17a, 19). However, several differences between these two enzymatic activities are apparent. In the absence of gRNA, the exonuclease activity is reduced but still removes all 3′ U's (Fig. 1B) while TUTase adds many U's in addition to the preferential addition of a single U 17, 24; Ernst et al., unpublished). Thus, the exonuclease specificity reflects the number of unpaired 3′ U's on the 5′ pre-mRNA and hence is unaffected by the absence of gRNA. The specificity of U addition reflects the ability of added U's to base pair with the gRNA and hence is affected by the absence of gRNA. The exonuclease activity is specific for unpaired U's (Fig. 4), while gRNA-specified U addition requires stabilization by base pairing. The presence of the 3′ pre-mRNA fragment along with the gRNA enhances exonuclease specificity, but overall activity remains unaffected (Fig. 1B). In contrast, their presence greatly increases both the specificity and the overall efficiency of the addition of more than one U. These properties probably reflect substrate recognition differences between the two activities. Indeed, as one might expect, substrates which have optimal efficiency and accuracy most closely resemble those which occur in vivo, i.e., a U overhang for U removal and a gap for U addition. The dramatic reduction of overall exonuclease and TUTase activities with the absence of the 5′ phosphate of the 3′ pre-mRNA fragment (Fig. 3C) may reflect a charge requirement for substrate binding, catalysis, or enzyme or complex conformation. Overall, the differential effects on exonuclease and TUTase activities due to the presence of the 3′ pre-mRNA fragment and/or gRNA suggest a combination of differential substrate recognition (binding) and specificity determinants.

The gRNA may indirectly specify the number of U's that are removed during deletion editing. It appears to primarily direct endonucleolytic cleavage and, hence, editing site selection, while U-specific exonuclease activity removes unpaired U's, thus providing some specificity (4). The gRNA may also contribute to specificity by enhancing ligation of the 5′ fragment containing the appropriate number of U's with the 3′ pre-mRNA fragment as a consequence of gRNA interactions with the pre-mRNA fragments and the editing complex. However, a base pair between mRNA and gRNA directly upstream of the ES is not required for ligation (10, 22a). The gRNA appears to play a more direct role in specifying the number of added U's by base pairing with the added U's (17, 17a, 22a).

The U-removal activity is not dependent on ATP. However, ATP enhanced ligation primarily of the correct U-removal product, in contrast to what occurred in precleaved or full-round insertion editing, where ATP primarily enhanced ligation of the input RNA (with no added U's) (7, 17). This enhanced ligation may have been due to differential utilization of the two editing complex RNA ligases (25, 28, 32, 34). Cruz-Reyes et al. (7) reported that endonucleolytic cleavage is dependent on adenosine nucleotides in deletion editing but not insertion editing. This may reflect the presence of more than one endonuclease activity. Indeed, at least four proteins in the editing complex share substantial sequence similarity (29). Hence, the editing complex may contain multiple endonucleases, exonucleases, and TUTases in addition to the two known RNA ligases (25, 28, 32, 34). Each set of enzymes with a common activity may have diverged from a common ancestor and may have retained similar catalytic capabilities while acquiring different substrate preferences. Thus, studies of exonuclease activity in the editing complex may examine the composite activity of multiple U-specific exonucleases. The increased activity with gRNA present may be catalyzed by one enzyme, and the low activity without gRNA may be catalyzed by another, although the different activities may be due to conformers of the same protein differentially bound within the complex. The two distinct activities may reflect U removal during deletion editing and a trimming off of excess U's that are added during insertion editing (5). The fact that the level of U removal activity was greater than that of U addition

activity in the presence of UTP (data not shown), even with U's that were able to pair with A's in gRNA (17a; Ernst, unpublished) again suggests that exonuclease may contribute to the specificity of U addition. We were unable to explore this by using phosphorothioate U analogs, since we have seen that the exoUase will remove the (*Sp*)-α-thio-U added by TUTase (Ernst, unpublished). An exoUase may also regulate the 3′ U tail lengths of gRNA (24) and kinetoplastid mt rRNA (1, 26).

Several 3′ exoribonuclease activities have been partially purified from *L. tarentolae* mitochondria (2), one of which is specific for U removal and has characteristics similar to the activities described here. The gene(s) for the exoUase(s) has not yet been identified, although the identification of the protein components and corresponding genes of the editing complex suggests that this will occur soon (28, 29). Nucleotide-specific exonuclease activities are unusual. An adenylate-specific 3′ exonuclease degrades poly(A) tails of mRNAs (21, 22), and *Escherichia coli* RNase E contains an A- and U-specific exoribonuclease activity under certain conditions (16). The multiprotein exosome contains several 3′ exoribonucleases (39), and the exosome of *T. brucei* lacks some enzymes that are present in that of *Saccharomyces cerevisiae* and has several homologs to enzymes of *E. coli*, whose exonucleases are not found in a multiprotein complex (11, 39). A relationship of the exoUase to other 3′ exonucleases, and perhaps to editing-associated TUTase, may provide some insight into its evolutionary origin and, hence, the origin of RNA editing.

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