Trypanosome U-deletional RNA editing involves guide RNA-directed endonuclease cleavage, terminal U exonuclease, and RNA ligase activities

JORGE CRUZ-REYES AND BARBARA SOLLNER-WEBB

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

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ABSTRACT We have studied the mechanism of accurate *in vitro* RNA editing of *Trypanosoma brucei* ATPase 6 mRNA, using four mRNA–guide RNA (gRNA) pairs that specify deletion of 2, 3, or 4 U residues at editing site 1 and mitochondrial extract. This extract not only catalyzes deletion of the specified number of U residues but also exhibits a novel endonuclease activity that cleaves the input pre-mRNA in a gRNA-directed manner, precisely at the phosphodiester bond predicted in a simple enzymatic model of RNA editing. This cleavage site is inconsistent with a chimera-based editing mechanism. The U residues to be deleted, present at the 3' end of the upstream cleavage product, are then removed evidently by a 3' U-specific exonuclease and not by a reverse reaction of terminal U transferase. RNA ligase can then join the mRNA halves through their newly formed 5' P and 3' OH termini, generating mRNA faithfully edited at the first editing site. This resultant, partially edited mRNA can then undergo accurate, gRNA-directed cleavage at editing site 2, again precisely as predicted by the enzymatic editing model. All of these enzymatic activities cofractionate with the U-deletion activity and may reside in a single complex. The data imply that each round of editing is a four-step process, involving (i) gRNA-directed cleavage of the pre-mRNA at the bond immediately 5' of the region base paired to the gRNA, (ii) U deletion from or U addition to the 3' OH of the upstream mRNA half, (iii) ligation of the mRNA halves, and (iv) formation of additional base pairing between the correctly edited site and the gRNA that directs subsequent nuclease cleavage at the next editing site.

RNA editing in trypanosomes is a fascinating form of transcript maturation in which U residues are inserted into, and less frequently deleted from, primary mitochondrial transcripts to generate mature mRNAs (for reviews, see refs. 1–5). The editing, which proceeds from 3' to 5' in the mRNA, can lead to very extensively edited messages, with U residues posttranscriptionally added at more than 100 different sites to comprise more than half the mature coding region. This unprecedented form of transcript maturation is directed by short guide RNAs (gRNAs), which contain an anchor region that can specifically base pair with the appropriate pre-mRNA, a region complementary to the edited version of the mRNA that directs the final U insertions and deletions, and a 3' nonencoded U tract (6, 7). Trypanosome mitochondria also contain gRNA–mRNA chimeric molecules in which the gRNA is covalently joined through its 3' oligo U tract to the downstream half of the mRNA at an editing site; these chimeras have been considered likely intermediates in an editing reaction in which the U residues are provided by the gRNA tail (8). Furthermore, in analogy with mRNA splicing and self-catalytic splicing, this

RNA editing has been proposed to use transesterification reactions (refs. 8 and 9; Fig. 1*B*), and that model has been very well accepted (11, 12).

Trypanosome mitochondria also contain an endonuclease that cleaves several pre-mRNAs in the vicinity of the first editing site (13) and an RNA ligase (14), so the same intermediates and partially edited mRNAs as proposed in the transesterification model could instead form by an enzymatic route (Fig. 1*C*). Although an initial report (15) that chimera formation in mitochondrial extract appeared not to require the editing site endonuclease seemed to disfavor this enzymatic model, cytochrome b (CYb) gRNA–mRNA chimera formation has since been shown to involve endonuclease and RNA ligase (16–18). However, those results do not demonstrate that the complete *in vivo* RNA editing reaction occurs by an enzymatic route, in part because the junction site of the CYb chimera formed *in vitro* is not at precisely the same mRNA residue as the chimera formed *in vivo* (19). The true editing process might not even use a chimeric intermediate. Indeed, RNA editing was originally envisioned to occur by an enzymatic, chimera-independent mechanism involving gRNA-directed nuclease cleavage of the mRNA, U addition or removal by the mitochondrial terminal U transferase (TUTase) or an exonuclease, and RNA ligation (ref. 6; Fig. 1*A*), but this model became substantially overshadowed by chimera-related mechanisms.

It recently became possible to accurately re-create an entire round of gRNA-directed U-deletional editing of ATPase subunit 6 (A6) pre-mRNA *in vitro* (20). This is a major advance in the study of RNA editing, because the accuracy of the *in vitro* reaction suggests that it mimics the *in vivo* editing mechanism. However, the data in that report (20) did not eliminate any of the three proposed mechanisms of editing.

We have examined the A6 U-deletion activity and found that it cofractionates with an endonuclease, a terminal U exonuclease, TUTase, and RNA ligase activities. This novel endonuclease activity cleaves A6 pre-mRNA in a gRNA-directed manner at editing site 1 (ES1), precisely as proposed for the endonuclease in the original enzymatic model of editing (ref. 6; Fig. 1*A*) and at a site that is inconsistent with the chimera-based mechanism (Fig. 1*C*). The upstream mRNA region then loses its 3' terminal U residues, evidently by a 3' U-specific exonuclease. Subsequent rejoining of the upstream and downstream mRNA halves by RNA ligase will then generate a complete, accurate, gRNA-directed cycle of editing. Furthermore, this editing product supports a second round of accurate, gRNA-directed cleavage precisely at ES2, a U-insertional editing site, whereas any inaccurately edited molecules are subject to re-editing at ES1.

FIG. 1. Models of U-deletional editing. RNA editing has been proposed to occur through a direct enzymatic route (6), with the gRNAs possibly serving to select the correct U-deleted product (10) (A); a transesterification-based scheme involving a gRNA-mRNA chimeric intermediate (8, 9) (B); and an enzymatic scheme involving a chimeric intermediate (4) (C).

MATERIALS AND METHODS

In Vitro RNA Production and Labeling. Pre-edited A6 wild-type (wt) mRNA (73 nt, with four U residues at ES1) and +1 mRNA (74 nt, with five U residues at ES1) were transcribed from PCR-generated templates amplified from A6/TAG and A6/TAG.1 DNA, kindly provided by K. Stuart and S. Seiwert (20), using primers T7A6 (5'-GTAATACGACTCACTATAGGAAAGGTTAGG-3') and A6-RT (20). ES1 edited (ES1_{ed}) mRNA was transcribed from a PCR-mutagenized template prepared using A6-TAG DNA and primers T7A6 and A6ES1_{ed} (5'-GATGCCAGGTAAGTATCTATAACTCCAATCACAAC-3'). PCR were assembled at 94°C (5 min) with polymerase added at 65°C, followed by 30 cycles of 94°C (45 sec), 41°C (45 sec), and 72°C (45 sec). The wt gRNA gA6[14] and the ΔG derivative gRNA were synthesized from cloned templates kindly provided by the Stuart laboratory (20). CYb gRNA was synthesized as described (16). All RNAs were prepared using T7 RNA polymerase and ³H-CTP, and after purification from 6% or 8% polyacrylamide/8 M urea gels, they were quantitated by scintillation counting. Where indicated, RNAs were labeled at the 3' end with [5'-³²P]PCP or labeled at the 5' end with T4 kinase and again gel purified. The A6 mRNA forms two bands (*n* and *n* + 1 nt), the shorter of which was used; the A6 gRNA transcripts have 3' U tracts of ≈12–15 nt. Enzymes were from BRL, and nucleotides were from Sigma.

Reactions in Mitochondrial Extract. Mitochondrial extract (2 × 10¹⁰ cell equivalents per ml) was prepared from procyclic *T. brucei* strain TREU 667 and fractionated by velocity centrifugation (15) for 8 hr, yielding 24 fractions of 0.5 ml. Catalase (at 11S) and thyroglobulin (at 19S) were standards. The 10-μl reactions in MRB buffer [25 mM Tris-HCl, pH 8/60 mM KCl/10 mM Mg(OAc)₂/1 mM EDTA/0.5 mM DTT/5% glycerol] contained 20–50 nM pre-mRNA, 100–150 nM gRNA, 1 μl of mitochondrial extract or 5 μl of gradient

fraction, and 3 mM ATP or ATP analogs. ADP supports gRNA-directed cleavage more efficiently than ATP and was used in place of ATP for Figs. 3B through 6. Following incubation at 25°C for 60 min, reactions were phenol extracted and ethanol precipitated in the presence of 1 μg of glycogen. U deletion was analyzed using reverse transcriptase (RT) and the A6-RT primer (20) or A6-RT2 primer (5'-GATGCCAGGTAAGTATTCTATAACTCC-3'). RNA cleavage was directly assessed using 5' or 3' end-labeled input pre-mRNA, RNA ligase was assessed using 5' end-labeled input pre-mRNA, and chimera formation was assessed using 5' end-labeled gRNA (17). All analyses were on 8% or 9% polyacrylamide/8 M urea gels; *Hpa*II-digested pBR-322 or the same substrate RNA digested using an RNase kit (United States Biochemical) or using nuclease P1 (BRL) [1200 units/ml in 23 mM sodium phosphate, pH 9/2 mM ZnCl₂/1 mM EDTA/6.6 M urea/90 μg/ml tRNA at 50°C (unpublished data)] were used as markers. Pyrophosphate and pyrophosphatase were from Sigma.

RESULTS AND DISCUSSION

Specific U Deletion. The first editing modification of A6 pre-mRNA, a transcript that will be extensively edited throughout its coding region (22), is U deletion from ES1 (Fig. 2A). A trypanosome mitochondrial extract has been shown to accurately catalyze this U deletion using wt or one-base-mutated (+1) A6 pre-mRNA in combination with wild type (wt) or one-base-mutated (ΔG) A6 gRNA (20); these gRNA-mRNA pairs direct deletion of two, three, or four U residues from ES1 of the pre-mRNA (the boldface U residues in Fig. 2B). This U deletion can be assessed by RT analysis using a radiolabeled primer and ddTTP (20) to terminate elongation at the A residue of the mRNA just 5' of the editing site (Fig. 2C Left). The deletion of four U residues from +1 mRNA directed by ΔG gRNA is shown in Fig. 2D (lane 2); the other three gRNA-mRNA pairs also yield RT extension bands of the expected size (ref. 20; Fig. 2B; data not shown). Also, the U deletion does not occur in the absence of a gRNA (Fig. 2D, lane 1) or in the presence of heterologous gRNA (Fig. 2D, lane 7), confirming its gRNA specificity.

Although the observed short RT extension band (Fig. 2D) requires both mRNA and gRNA and has a size indicative of accurate U deletion, a short extension band of the same size could also be generated from a different molecule, a gRNA-mRNA chimera that lacks the entire 3' U tract of the gRNA (Fig. 2C Right). Because there is precedent for such deleted chimeras forming in mitochondrial extract (19, 24), we assessed A6 chimera formation in our reactions using radiolabeled gRNA and unlabeled pre-mRNA. A6 gRNA-mRNA chimeras are formed extremely inefficiently in our reactions, at <1/50 the abundance that would be needed to generate an extension band of the same intensity as that shown in Fig. 2D (data not shown). Also, the detected A6 chimeras retain the U tract of the gRNA and, thus, would form longer extension bands than the input mRNA (Fig. 2C Right). Finally, when the RT reactions are performed using ddCTP, the extension band is 1 nt longer than that generated using ddTTP, indicating that it arises from U-deleted mRNA and not from a chimera (Fig. 2C, bottom lines). Thus, the novel short band in the RT assay is diagnostic for A6 U deletion.

Role for RNA Ligase. Because *in vitro* chimera formation has recently been shown to use an endonuclease and RNA ligase (16–19), we examined whether RNA ligase may function in the gRNA-directed U deletion of A6 mRNA. Consistent with this possibility and the known mechanism of RNA ligase involving ATP α-β bond hydrolysis and pyrophosphate release, the A6 U deletion requires ATP and does not function with AMP-CPP (ref. 20; Fig. 2D, lanes 8 and 12). If U deletion does require RNA ligase, the reaction should also be end-product

FIG. 4. gRNA-directed A6 mRNA cleavage at ES1. The indicated 3' end-labeled mRNA and gRNA were incubated with the 20S gradient fraction or with alkali (OH) or *B. cereus* RNase (Bc), or with subsequent calf intestinal phosphatase (CIP) treatment.

terminus, for a 5' P causes nucleic acid to migrate $\approx 1/2$ nt faster than the corresponding fragment with a 5' OH terminus (25). Confirming that the A6 endonuclease generates 5' P termini, the cleaved pre-mRNA precisely comigrates with the marker RNA when both are treated with phosphatase before electrophoresis (Fig. 4, lanes 13 and 14) or when marker RNA is prepared using nuclease P1, which generates 5' P termini (data not shown). Production of 5' P termini by this gRNA-directed mitochondrial endonuclease (Figs. 3C and 4) is notable, because 5' P termini are substrates for the mitochondrial RNA ligase (16–18), consistent with a role for this nuclease in an enzymatic editing mechanism.

For each of the four A6 mRNA–gRNA pairs (Fig. 2B), the precise position of the endonuclease cleavage site in the 3' end-labeled pre-mRNA was mapped using RNase sequencing standards prepared from the same labeled pre-mRNA (Fig. 4). The cleavage position varies within the U tract of ES1, depending on the gRNA. With wt gRNA, the predominant downstream fragments from both wt mRNA and +1 mRNA retain two U residues of their original U₄ or U₅ tracts, respectively (Fig. 4, lanes 3 and 8), whereas with the ΔG gRNA, the predominant downstream fragments of both pre-mRNAs retain one U residue of their U tracts (lanes 4 and 9). These cleavages are precisely at the first phosphodiester bond of the mRNA 5' of the region that can base pair with the gRNA (indicated by the arrow in Fig. 2B). Thus, in all four mRNA–gRNA pairs, the endonuclease cleavage is positioned so that the number of U residues that the gRNA specifies to be retained are located on the downstream mRNA fragment, whereas the number of U residues to be removed (two, three, or four) are on the upstream mRNA fragment. This cleavage pattern (Fig. 2B) is exactly as predicted by the direct enzymatic model of editing (Fig. 1A).

U Removal from the Upstream mRNA Fragment. The gRNA-directed endonuclease activity can also be detected using 5' end-labeled A6 pre-mRNA and unlabeled gRNA, in which case the upstream rather than the downstream mRNA half is observed. Notably, the upstream mRNA fragment is not a single discrete size like the downstream fragment; rather, it varies by a few nucleotides, with the ends again mapping to the U tract of ES1 (Fig. 3D). Furthermore, the extent of size heterogeneity differs for the different mRNA–gRNA pairs. There are four major upstream fragment sizes with the wt mRNA– ΔG gRNA pair (Fig. 3D), four with the +1 mRNA–wt gRNA pair (Fig. 5A, lane 3), five with the +1 mRNA– ΔG gRNA pair (Fig. 5A, lane 4), and three with the wt mRNA–wt gRNA pair (data not shown). Thus, the number of 5' fragment sizes is one more than the number of U residues that are to be deleted (compare with Fig. 2B).

The upstream mRNA fragments were sized relative to digests of the same 5' end-labeled A6 mRNA generated by nuclease P1 under conditions in which cleavage is preferential for G residues (Fig. 5B; nuclease P1 yields 3' OH termini, like the extract endonuclease, whereas the 2'-3' cyclic phosphate

FIG. 5. U removal at the 3' end of the upstream cleavage fragment. All reactions used the 20S gradient fraction, ΔG gRNA unless indicated, and were assayed as in Fig. 3. (A and B) The 5' fragment from 5' end-labeled +1 mRNA incubated with the indicated gRNA and 20S fraction or with the indicated RNases. *Phy M* (PM), T1, *B. cereus* (Bc) RNases, and alkali (OH) cleave 3' of U+A, G, U+C, and all residues, respectively, leaving 2'-3' cyclic P termini; nuclease P1 cleaves 3' of G residues, leaving 3' OH termini, like the extract endonuclease. (C) The 5' fragment from 5' end-labeled wt mRNA was assayed at various times. Lane 6 includes 2 mM UTP. (D) RNA ligase assayed in the reactions of Fig. 5C, lanes 5 and 6. (E and F) U deletion from +1 mRNA and ΔG gRNA assayed at various times. Two millimolar NTP additions are indicated. (G) The 5' mRNA fragment (Upper) and RNA ligase (Lower) were assayed using 5' end-labeled wt mRNA and the indicated pyrophosphate (mM) and pyrophosphatase (units/ml) amounts.

termini generated by the RNases usually employed for sequence analysis cause anomalous migration in electrophoretic sequencing gels; unpublished data). For each mRNA–gRNA pair, the largest of the upstream mRNA fragments corresponds to cleavage of the phosphodiester bond identified using the 3' end-labeled mRNA (Fig. 4), the shorter bands correspond to molecules sequentially lacking 3' terminal U residues, and the shortest band corresponds to the upstream mRNA fragment from which all the 3' terminal U residues were removed (Fig. 5B; data not shown; compare with Fig. 2B).

Examining kinetics, the largest fragment reaches relatively steady levels at early times of reaction, whereas the smallest fragment becomes far more abundant at later times of reaction (Fig. 5C). The increase in the total amount of these upstream cleavage products during the time course corresponds to the

increase in the amount of the downstream cleavage product (data not shown). These results indicate that there is an initial endonuclease cleavage of the mRNA at the phosphodiester bond immediately 5' to the residues that are base paired with the gRNA (Fig. 2*B*, arrow), and then the terminal U residues at the 3' end of the upstream mRNA fragment are removed. The 3' removal stops at the end of the U tract (Fig. 5*A* and *C*). These are precisely the activities proposed in the direct enzymatic model of RNA editing (Fig. 1*A*).

Addition of UTP to the extract reaction inhibits accumulation of the upstream mRNA fragment that completely lacks the 3' terminal U residues (Fig. 5*C*, lane 6). This inhibition is specific for UTP and is not similarly observed with GTP, CTP, or ATP in the reaction (data not shown). Similarly, production of the correctly edited mRNA is also inhibited by addition of UTP but not by the other NTPs (Fig. 5*F*; data not shown). In contrast, neither the pre-mRNA cleavage nor RNA ligase is inhibited by UTP (Fig. 5*C* and *D*; data not shown). These results support an editing model in which complete removal of the U residues from the upstream mRNA fragment is a prerequisite for the correct U-deleted mRNA. Also consistent with the U-deleted mRNA deriving from the upstream mRNA fragment lacking the 3' U residues, the kinetics of generating this fragment (Fig. 5*C*) parallel the kinetics of U deletion (Fig. 5*E*). Furthermore, the 3' U removal generates 3' OH termini (Fig. 5*B*), which are proper substrates for RNA ligase.

The observations that removal of 3' terminal residues from the upstream mRNA fragment stops at the end of the U tract whether this segment is initially 4, 3, or 2 nt in length (Fig. 5*A* and *C*; data not shown) and that these phosphodiester bonds are only cleaved in molecules where the initial ES1 cleavage reaction has already taken place (Fig. 3*C* and *E*) suggest that this removal is catalyzed by an exonuclease and that this exonuclease is U-specific. A U-specific exonuclease has been previously implicated in trypanosome mitochondrial extract (19), and we similarly observed input gRNA molecules slowly losing their 3' terminal U residues with all degradation stopping at the end of the U tract (data not shown).

One simple explanation for a U-specific exonuclease activity would be the TUTase enzyme working in reverse, because its pyrophorolysis would convert U-terminated RNA and pyrophosphate into an RNA that is shorter by one residue at the 3' end and UTP. However, that does not appear to be the case. A TUTase reverse reaction should be stimulated by pyrophosphate addition and inhibited by pyrophosphatase, yet the 3' U removal exhibits neither of these attributes (Fig. 5*G* Upper, lanes 4, 5, 7, and 8). Internal controls demonstrate that the pyrophosphate and pyrophosphatase were active (Fig. 5*G* Lower). These data imply that the 3' terminal U removal is catalyzed by a distinct nuclease and not by TUTase.

The A6 pre-mRNA cleavage and 3' terminal U removal reactions occur in the absence of RNA ligase activity (Fig. 5*G*, lanes 4 and 5). This is also predicted by the direct enzymatic editing model (Fig. 1*A*), in which RNA ligase acts after the endonuclease and U exonuclease in each cycle of editing.

The 3' terminal U-removal activity cosediments at ≈ 20 S with the gRNA-directed endonuclease activity, the RNA ligase activity, and the complete U-deletion activity (Fig. 3*D*). If the 3' U-removal activity had a different sedimentation coefficient than the endonuclease, the relative amounts of the various size upstream fragments would differ across the gradient, which they do not (Fig. 3*D*). This cosedimentation further supports the existence of an "editosome" complex that contains all the activities involved with U-deletional RNA editing.

Editing Verification and Specific Cleavage to Initiate a Round of U-Insertional RNA Editing. The product of the gRNA-directed U deletion of A6 mRNA is correctly edited at ES1 (ES1_{ed} mRNA). The duplex between the mRNA and the gRNA would then "zip up" by one residue, so the phosphodiester bond of the mRNA immediately adjacent to the enlarged

base paired region would now be ES2 (Fig. 6*A* Left), a site of U-insertional editing. If U-insertional editing also proceeds through the direct enzymatic route (Fig. 1*A*) and if the editing endonuclease acts on other editing sites of A6 mRNA like it does at ES1, the wt gRNA should direct cleavage of ES1_{ed} mRNA precisely at ES2 (Fig. 6*A* Left). This is exactly what was observed (Fig. 6*B*, lane 3). This result suggests that the mitochondrial extract might be able to catalyze U-insertional editing at ES2; it also provides additional evidence that the gRNA-directed editing endonuclease cleaves with the specificity predicted by the direct enzymatic model (Fig. 1*A*).

If the first round of editing at ES1 had not deleted the exact number of U residues specified by the gRNA, then the duplex between the mRNA and the gRNA could not zip up to ES2. For instance, if wt mRNA had deleted only two and not three U residues under the direction of ΔG gRNA, the resultant base pairing would be as in Fig. 6*A* (Right), and cleavage by the editing endonuclease would be expected to occur at ES1 between the two remaining U residues. This is also precisely what was observed (Fig. 6*B*, lane 4). Thus, the specificity of the gRNA-directed editing endonuclease could serve to check the previous round of editing modification. If the correct number of U residues had not been deleted, that previous editing site would be recleaved for another round of editing (as in Fig. 6*B*, lane 4), but if the editing was correct, the next editing site would be cleaved (as in Fig. 6*B*, lane 3).

CONCLUSIONS

We show that *in vitro* U-deletional RNA editing at the first editing site of A6 pre-mRNA occurs by a direct enzymatic mechanism (Fig. 1*A*). This involves (i) pre-mRNA cleavage by a novel gRNA-directed endonuclease precisely at the 5' border of the residues that are base paired to the gRNA, (ii) removal of the extra U residues from the 3' end of the upstream mRNA half, (iii) ligation of this processed upstream mRNA half to the downstream mRNA half, and (iv) zipping up of the gRNA-mRNA base pairing at the correctly edited site to prevent further cleavage at that position and direct cleavage to the next (5') site. This mechanism is largely identical to the initially proposed model for gRNA-directed editing (6), with the added notion that gRNAs may serve to select and preserve correctly edited sites rather than to initially direct the extent of U deletion (10). As this manuscript was being submitted for review, Seiwert, Heidmann, and Stuart (23) published com-

FIG. 6. gRNA-directed cleavage at the U-insertional editing site ES2 on mRNA edited at ES1. (A) Potential base pairing of ES1_{ed} mRNA and wt (Left) and ΔG (Right) gRNA. (B) The 3' end-labeled ES1_{ed} mRNA was incubated with the indicated gRNA in the 20S gradient fraction or with the indicated RNase. Sizing analysis as in Fig. 5*B* demonstrates that the cleavage positions correspond to the thick arrows in A; there is no cleavage at the position indicated by the thin arrow.

plementary data leading to this same general mechanism of U-deletional editing.

We present a number of lines of evidence that the activities described above constitute the U-deletion process. The endonuclease cleavage is gRNA-directed and occurs at precisely the site expected for the direct enzymatic editing mechanism on a total of six different mRNA–gRNA pairs (Figs. 4 and 6). Also, this nuclease generates 5' P and 3' OH termini, and such termini are substrates for RNA ligase and for TUTase (Fig. 4). The 3' exonuclease appears specific for U residues and, thus, stops when the “correct” number of U residues have been removed (Fig. 5A–C); it also generates 3' OH termini, which are proper substrates for RNA ligase (Fig. 5B). In addition, its kinetics of action and the specific inhibition of product accumulation by UTP correspond well to the kinetics and inhibition of the U-deletion reaction (Fig. 5C, E, and F). Furthermore, RNA ligase exhibits sensitivities to ATP analogs and to pyrophosphate that mimic the sensitivities of the U-deletion reaction, and the ligase must act after rather than before the endonuclease and U-deletion steps (Figs. 2D and 5G). These data suggest that the *in vitro* editing occurs by the mechanism shown in Fig. 1A. Furthermore, because of the accuracy of the *in vitro* editing, it is likely that these reactions are representative of the *in vivo* editing mechanism.

All these enzymatic activities cosediment with the U-deletion activity at ≈ 20 S, which is considerably faster than free proteins of the extract. The implication is that the gRNA-directed endonuclease, the U-specific 3' exonuclease, and the RNA ligase (Fig. 3) of the U-deletional editing mechanism, as well as the TUTase (ref. 15; L. Rusché and B.S.W., unpublished observation) that is proposed to act in the analogous U-insertional editing model (5), are present in a macromolecular complex. This complex could be an editosome that helps hold the relevant components together in high local concentration. The editing endonuclease we have detected, which is gRNA-directed and cleaves the mRNA immediately adjacent to the base paired region (Figs. 2B and 4), appears to be different than earlier detected endonucleases that cleave pre-edited mRNA in a gRNA-independent manner and do not cleave immediately adjacent to a duplex region (13, 19, 21).

The ability of the editing endonuclease to accurately cleave not only at the U-deletional site, ES1 (Fig. 4), but also at the U-insertional site, ES2 (Fig. 6), suggests that this nuclease activity may be involved in both U-insertional editing and U-deletional editing. Its ability to discriminate an mRNA that is misedited by only one U residue, relative to the base paired gRNA (Fig. 6), further suggests that this nuclease may enable proofreading of the editing. Curiously, although the 5' mRNA halves present in the reaction contain varying numbers of 3' U residues (Figs. 3 and 5), the U-deleted product that accumulates derives only from ligation of the 5' mRNA half that has lost all its 3' U residues (Fig. 2B). This is particularly striking under conditions where incomplete U removal is favored (Fig. 5C, lane 6), but mRNA products bearing partial U-deletions do not similarly accumulate (Fig. 5F, lane 2). Thus, either the editing complex only allows ligation of the fully processed 5' mRNA fragment or partially processed 5' mRNA halves also

become ligated but the product is rapidly recleaved. The kind of gRNA-directed cleavage shown in Fig. 6B, lane 4, could serve this function, and such cleavage may be favored on newly ligated molecules that are still bound to the editing complex.

Finally, it is important to stress that the position the endonuclease cleaves A6 mRNA (Fig. 4) is not consistent with the enzymatic model of editing involving a gRNA–mRNA chimeric intermediate. In that model (Fig. 1C), the U residues that will be deleted are contained on the downstream mRNA fragment, for transfer to the gRNA through the chimera. However, the endonuclease we observe places the U residues that will be deleted on the upstream mRNA fragment, which is instead supportive of the direct enzymatic model of editing (Fig. 1A).

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