layered structure would also help to reduce some of the practical issues associated with casting a large monolithic piece of low–phonon energy glass, as these materials have a low thermal conductivity and are difficult to fabricate in pieces thicker than a few inches. Such a structure would enable large displays having several inches on a side to be manufactured.

Conclusion. The solid-state, 3D display technology described here can be driven entirely with commercially available laser diodes and scanning systems and can be powered directly from 120-V, 60-Hz electrical outlets. The display operates at room temperature and is viewable under typical ambient room lighting conditions. Current efforts are under way to implement a more fully integrated system architecture and to increase upconversion efficiencies with improved material systems. We believe that this technique offers a viable approach for presenting real-time, multidimensional information to a multitude of viewers, independent of viewing perspective, with no obstructed viewing regions and no need for special viewing eyewear.

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RNA Editing: A Mechanism for gRNA-Specified Uridylate Insertion into Precursor mRNA

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In the mitochondria of trypanosomatid protozoa the precursors of messenger RNAs (pre-mRNAs) have their coding information remodeled by the site-specific insertion and deletion of uridylate (U) residues. Small trans-acting guide RNAs (gRNAs) supply the genetic information for this RNA editing. An in vitro system was developed to study the mechanism of U insertion into pre-mRNA. U-insertion editing occurs through a series of enzymatic steps that begin with gRNA-directed pre-mRNA cleavage. Inserted U's are derived from free uridine triphosphate and are added to the 3′ terminus of a 5′ pre-mRNA cleavage product. gRNA specifies edited RNA sequence at the subsequent ligation step by base pairing–mediated juxtaposition of the 3′ cleavage product and the processed 5′ cleavage product. gRNA/pre-mRNA chimeras, purported intermediates, seem to be abortive end products of the same reaction.

Most mitochondrial pre-mRNAs in trypanosomes undergo RNA editing, a form of RNA processing that markedly alters their U content and consequently changes their coding information (*1*). In *Trypanosoma brucei*, RNA editing inserts a total of 3030 U's into hundreds of editing sites in 12 different pre-mRNAs. RNA editing also removes U's from specific positions, but at a 10-fold lower frequency. Both types of changes in informational content are specified by small [~ 60 nucleotides (nt)], trans-acting RNAs termed gRNAs. The mitochondrion of *T. brucei* contains numerous gRNAs, each of which is complementary to a region of an edited mRNA and which collectively are diverse enough in sequence to specify all of the observed editing.

The exact mechanistic role of gRNAs in RNA editing is not well defined. Before U insertion and deletion, the 5′ portion of a gRNA is thought to form a short intermolecular duplex with its cognate pre-mRNA immediately 3′ of the region to be processed. Mismatched purines in the gRNA sequence adjacent to this duplex then specify U insertion, whereas mismatched U's in the adjacent pre-mRNA sequence are deleted. Both events extend the complementarity between gRNA and pre-mRNA to produce an uninterrupted intermolecular helix.

Elucidation of the biochemical mechanism of RNA editing and its component steps hinges on identifying the source of inserted U's. In alternative models of RNA editing, either free uridine triphosphate (UTP) (*2*) or nonencoded U residues at the 3′ end of gRNAs (*3*) have been suggested as the source of these residues.

We have developed a cell-free system that reproduces U-insertion editing in vitro and which therefore allows direct analysis of the mechanism of this reaction. (The insertion or deletion of U's at one editing site is referred to as the editing "reaction," although it occurs by a series of catalytic steps.) We compare this mechanism to that of the less frequent U-deletion editing previously examined (*4*).

U insertion is gRNA- and UTP-dependent. To study U insertion, we used a synthetic transcript based on adenosine triphosphatase 6 pre-mRNA (A6-eES1) (Fig. 1) (*5*) as an editing substrate. It corresponds to an RNA that is edited at only the 3′-most editing site (ES1) by the deletion of two U's. The gRNA termed gA6[14] (*6*) is predicted to direct the insertion of

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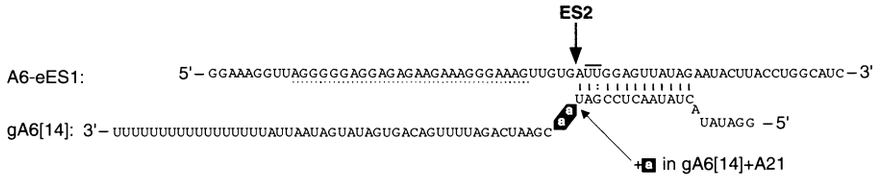

Fig. 1. ATPase 6 substrate RNA and gRNA. The 70-nt substrate RNA (A6-eES1) is shown engaged in an intermolecular anchor duplex with the 71-nt gRNA (gA6[14]) via Watson-Crick (vertical lines) and G:U (colon) base pairs. A purine-rich sequence in the substrate RNA (dotted underline) may base-pair to the 3′ oligo(U) tail of the gRNA. Editing site 1 (indicated by a solid horizontal line) contains the sequence present in the mature in vivo mRNA. Editing site 2 contains unedited sequence. Adenylates in gA6[14] and gA6[14]+A21, which predict the insertion of two or three U's into ES2, respectively, are outlined in black.

two U's into the penultimate editing site (ES2) of this substrate RNA (Fig. 1). To detect U insertion, we incubated end-labeled substrate RNA with gRNA in ~20S to 35S glycerol gradient fractions of *T. brucei* mitochondrial lysates (*7*) and analyzed the bulk RNA population by polyacrylamide gel electrophoresis to visualize potential editing intermediates and edited RNA.

Incubation of 3′ end-labeled substrate RNA (labeled B in Fig. 2A) with gRNA, UTP, and mitochondrial extract resulted in the accumulation of four RNA species (A, C, $D_{n=2}$, and $D_{n>2}$). When UTP was omitted, a fifth species, $D_{n=0}$, was formed. Species C was the size expected of substrate RNA into which two U's had been inserted (Fig. 2B), as would be predicted if gA6[14] directed correct editing at ES2 (Fig. 1). RNA sequencing of species C purified from preparative reactions with ribonuclease (RNase) T1, RNase U2, and RNase Phy M (Fig. 3, A and B) revealed that it was identical to the input RNA both 5′ and 3′ to ES2, but was altered by the insertion of two U's at this site. Thus, species C represents substrate RNA that is correctly edited at ES2.

As predicted by models of RNA editing (*1*), edited RNA was not formed if gRNA was omitted (Fig. 2B, lane 3). In contrast to U-deletion editing (*4*) and the predictions of some models of U-insertion editing (*3*, *8*), production of edited RNA also required inclusion of UTP in the in vitro reaction (Fig. 2B, lane 4). Neither 5′ or 3′ uridine monophosphate (UMP), cyclic UMP, or poly(U) could substitute for UTP (*9*).

Partial digestion of species A with RNase T1 indicated that it represents the 3′ portion of substrate RNA that had been generated by endonucleolytic cleavage at or near ES2 (Fig. 3A, lane 1). Substrate RNA cleavage at this site required gRNA, but not UTP (Fig. 2A), indicating that one function of the gRNA during in vitro U insertion is to direct endonucleolytic cleavage of the pre-mRNA.

This cleavage product migrated as if it were approximately 1 nt smaller than its expected size compared to RNase T1 (Fig. 2A) and hydroxyl ladders (*9*). This is the shift predicted if the cleavage product contains the additional negative charge contributed by a monophosphate (*4*). To determine the exact site of cleavage that produced species A, we performed run-off primer extension analysis (*7*) with oligonucleotide A6-RT (*10*) as a primer on species A purified from a preparative reaction (Fig. 3C). Comparison of the size of this run-off primer extension product to primer extension sequencing of the substrate RNA indicated that species A represents the 3′ half of the substrate RNA cleaved precisely at ES2 (arrow in Fig. 1). Thus, unlike the marker ladders, the 3′ cleavage product generated during the U-insertion reaction probably carries a 5′ monophosphate, as is the case during in vitro U-deletion editing (*4*) and with the *T. brucei* mitochondrial pre-mRNA cleavage activity reported previously (*11*).

Partial RNase T1 digestion of species $D_{n=0}$, $D_{n=2}$, and $D_{n>2}$ showed that they are all gRNA/substrate RNA chimeric molecules (chimeras) in which 5′ gRNA sequences are linked by various numbers of U's to the 3′ cleavage product of the substrate RNA [Fig. 3A (*9*)]. The nucleotide spacing between the 3′-most G derived from the gRNA and the 5′-most G derived from the substrate RNA suggests that two U's link the gRNA and substrate RNA sequences in chimera $D_{n=2}$. A greater number of U's link the two RNAs in the heterogeneous collection of chimeras labeled

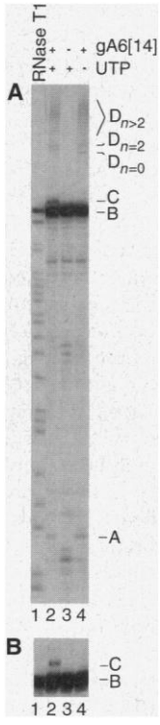

Fig. 2. UTP and gRNA dependence of processing. (**A**) Electrophoretic analysis (*7*) of synthetic [α-^{32}P]pCp 3′ end-labeled (*5*) substrate RNA partially digested with RNase T1 (lane 1) or used in in vitro reactions that included UTP and gRNA (lane 2), lacked gRNA (lane 3), or lacked UTP (lane 4). Input substrate RNA (B) and in vitro–produced species (A, C, $D_{n=0}$, $D_{n=2}$, and $D_{n>2}$) are indicated. (**B**) Analysis of the same samples as in (A) by electrophoresis for a longer period of time to enhance resolution of RNA species B and C.

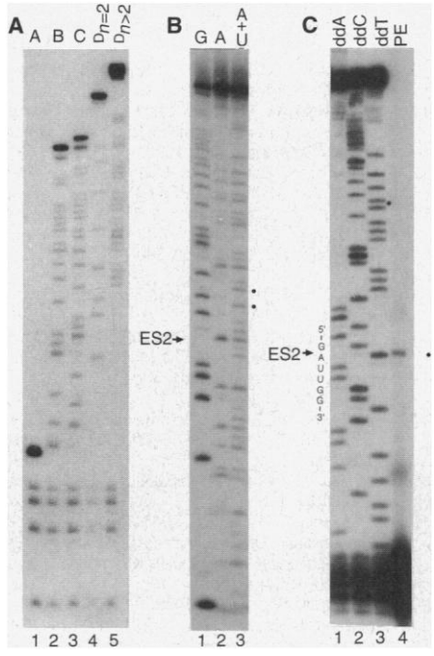

Fig. 3. Sequence characterization of processing products and edited RNA. (**A**) Electrophoretic analysis of RNA species A, B, C, $D_{n=2}$, and $D_{n>2}$ (lanes 1 to 5, respectively) gel-purified from preparative reactions like that in Fig. 2A, lane 2, after partial digestion with RNase T1. (**B**) Complete RNase sequencing of edited A6-eES1. Presumptive edited RNA generated in a preparative reaction containing 3′ end-labeled A6-eES1 and gA6[14] was excised after gel electrophoresis and subjected to partial digestion with RNase T1 (lane 1), RNase U2 (lane 2), or RNase PhyM (lane 3). The cleavage specificity of each RNase is indicated above each lane. ES2 is marked by an arrow and the inserted U's are indicated by dots. (**C**) Primer extension analysis of the 3′ product generated by gRNA-directed substrate RNA cleavage. The 3′ product generated by gA6[14]-directed cleavage of 3′ end-labeled A6-eES1 (species A) was isolated from a preparative reaction and subjected to run-off reverse transcription (*7*) after annealing 5′-radiolabeled oligonucleotide A6-RT (PE, lane 4). The length of this primer extension product was analyzed relative to reverse transcription sequencing of A6-eES1 with the same primer in reactions containing either dideoxyadenosine (lane 1), dideoxycytidine (lane 2), or dideoxythymidine (lane 3). ES2 is indicated.

$D_{n>2}$. No U residues are present at the gRNA/substrate RNA linkage site in chimera $D_{n=0}$ (9).

Free UTP is the source of the inserted U's. We investigated whether the UTP requirement reflected UMP addition to the 3′ end of the 5′ cleavage product by examining the products that resulted from incubation of 5′ end-labeled substrate RNA in the presence or absence of UTP (Fig. 4A). As expected from the 3′ end-labeling experiments (Fig. 2), a species the size of edited product was produced only when both UTP and gRNA were included in the incubation (Fig. 4A). When only gRNA was present, the 5′ cleavage product predicted from targeted cleavage of the substrate RNA at ES2 was generated (indicated by a dot in Fig. 4A, lanes 3 and 4). As predicted by the characterization of the 3′ cleavage product [Fig. 3 (9)] and previous studies (*4*, *11*), the 5′ cleavage product appears to terminate in a 3′ hydroxyl because it has a slightly slower mobility than expected relative to marker fragments produced by RNase T1 (which carry a 3′ phosphate).

Inclusion of UTP in a reaction that also contained gRNA resulted in production of RNAs that were 1, 2, and 3 nt larger than the 5′ cleavage product that is observed in reactions without UTP (compare lanes 3 and 4, Fig. 4A). Although molecules of the same size are apparent in reactions lacking gRNA, the larger products seen in lane 4 probably do not result from endonucleolytic cleavage, because gRNA protects the substrate RNA sequence in this region from cleavage (compare lanes 2 and 3, Fig. 4A). Thus, all three species in lane 4 likely represent the 5′ cleavage product with U's added to its 3′ end. Substrate RNA with two U's at ES2 is the predominant edited product (Figs. 2 and 4A). Because a prominent 5′ product containing two U's is not observed, it must be rapidly utilized if it exists. U addition to the 5′ cleavage product may not be specified by the gRNA. In this instance the 5′ cleavage product that (probably) has two U's at its 3′ end would be selected from the population of molecules containing different numbers of U's.

If the inserted U's are derived from free UTP, then the substrate RNA should become radiolabeled at ES2 if [α-^{32}P]UTP is included in the reaction. To test this hypothesis, we added [α-^{32}P]UTP to a reaction containing unlabeled RNA (Fig. 4B) (7). Gel electrophoresis of RNA collected from this reaction showed that molecules of many sizes were radiolabeled (9), probably by terminal uridylyl transferase (TUTase)–mediated U addition to the 3′ end of gRNAs or carrier RNA (or both), as well as U insertion into the substrate RNA. Molecules the size expected of edited product were excised and eluted from this gel. One-half of the samples that contained gRNA were digested with RNase H (*7*) in the presence of an oligonucleotide complementary to the 5′ end of the gRNA, and molecules the size of edited product were again isolated by gel electrophoresis. RNAs in all samples were then digested to completion with RNase T1.

If two U's were inserted into ES2 in the presence of gRNA, then radiolabeled fragments of 2 and 6 nt (derived from edited product) should be produced (Fig. 4B). The size-selected products resulting from in vitro editing reactions that included gRNA and that were not subjected to the subsequent RNase H treatment yielded three radiolabeled fragments upon complete RNase T1 digestion (Fig. 4C, lane 7). Two of these species comigrated with synthetic oligoribonucleotide markers corresponding to the expected ^{32}P-labeled fragments derived from RNase T1 digestion of an edited product containing two U's at ES2. These two fragments were produced only when substrate RNA was included in the reaction (lane 5) and persisted after RNase H–mediated destruction of gRNA (lane 6). Thus, radioactive 5′-UMP appears to be correctly inserted into ES2 of the substrate RNA in a gRNA-dependent manner during in vitro U-insertion editing. The largest fragment (26 nt) had the size expected of a 3′ terminal gRNA fragment with an additional U added to its 3′ end. It was absent in the sample treated with RNase H to destroy the gRNA (lane 6) and was absent if gRNA was omitted from the in vitro editing reaction (lane 3), indicating that it indeed represents a fragment derived from gRNA. The absence of other major labeled products in reactions that included gRNA and substrate RNA (lanes 6 and 7) indicates that ES2 is specifically targeted for processing among all of the internucleotide sites in the substrate RNA, in agreement with RNase sequencing of the edited RNA [Fig. 3B (9)].

Guide RNAs determine the number of inserted U's. A mutant gRNA with an additional adenylate in the region that directs processing at ES2 (gA6[14]+A21, see Fig. 1) (5) was used to investigate whether U insertion was determined by the sequence of the gRNA included in the reaction. This mutant gRNA should direct the insertion of three (rather than two) U's at ES2 if base-pairing interactions between the gRNA and the substrate RNA at the processing site control U insertion. Reactions that included this gRNA resulted in an RNA product that was 1 nt larger than

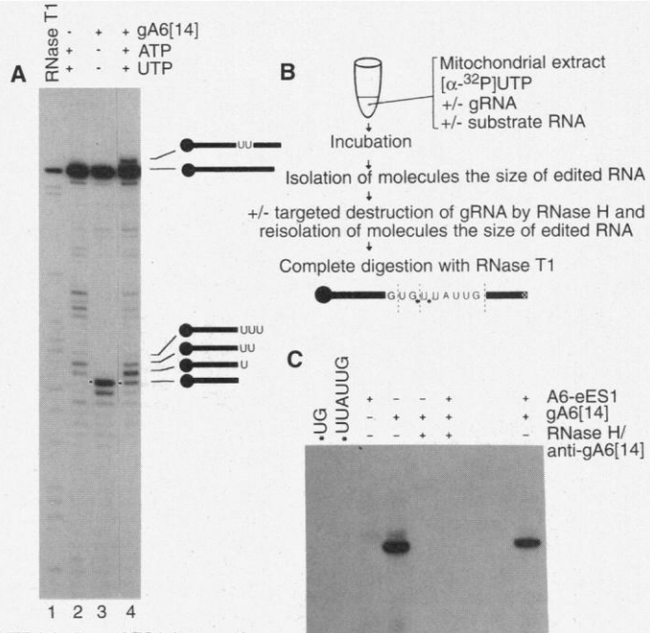

Fig. 4. The role of UTP. (**A**) Electrophoretic analysis of substrate RNA that was 5′ end-labeled with [α-^{32}P]GTP through use of guanylyl transferase and treated with periodate (to prevent addition of nucleotides to its 3′ end) (5) after in vitro incubation (lanes 2 to 4) or partial digestion with RNase T1 (lane 1). Reactions were performed as described (7) and included (lanes 3 and 4) or lacked (lane 2) gRNA, or lacked exogenously added ATP and UTP (lane 3). Products in lanes 3 and 4 that are 1, 2, and 3 nt smaller than the expected cleavage product probably result from 3′ exoribonuclease activity. (**B**) Schematic of experiment that examines [α-^{32}P]UTP labeling of ES2 (see text). Complete RNase T1 digestion of the substrate RNA (7) with two [α-^{32}P]UMP residues inserted into ES2 will produce the two labeled fragments demarcated with dashed vertical lines. (**C**) Analysis on a 20% polyacrylamide gel of [α-^{32}P]UMP-labeled RNA produced as outlined in (B). In vitro reactions (lanes 3 to 7) contained 20*S* to 35*S* glycerol gradient fraction of mitochondrial lysate, [α-^{32}P]UTP, unlabeled A6-eES1 substrate RNA (lanes 3, 6, and 7), and gRNA (lanes 4 to 7). Samples in lanes 5 and 6 were treated with RNase H to destroy gRNA. Oligoribonucleotides UG (lane 1) and UUAUUG (lane 2) that correspond to the sequences of the expected labeled T1 fragments were used as markers after labeling by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP.

that produced in reactions containing the wild-type gRNA (Fig. 5, A and B). RNase sequencing of this larger species after purification from a preparative reaction showed that it represents edited product with three U's at ES2 (9). Thus, genetic information specified by gRNAs is transferred to substrate RNA during in vitro U insertion.

The effect of gA6[14]+A21 on U addition to the 5′ cleavage product was examined in in vitro reactions performed with 5′ end-labeled substrate RNA. In the presence of adenosine triphosphate (ATP) and UTP, a population of molecules 1, 2, and 3 nt larger than the 5′ cleavage product was formed (compare lanes 2 and 3 in Fig. 5C). The size distribution of these products appears to be the same as those produced in a reaction containing the wild-type gRNA (Figs. 4A and 5C). Thus, different gRNAs do not seem to specify the number of U's added to the 5′ cleavage product. Both gRNAs direct initial endonucleolytic cleavage of substrate RNA to the same site (Fig. 5A), as would be predicted if the cleavage site is determined by the anchor duplex, which is the same with both gRNAs.

U insertion with a different gRNA/substrate RNA pair. To determine if U insertion would occur in vitro with a gRNA/substrate RNA combination other than gA6[14]/A6-eES1, we investigated U insertion at editing site 1 of the frameshift region of the *Crithidia fasciculata* ND7 pre-mRNA (ES1-Cf). This gRNA/substrate RNA pair was chosen based on the report of an in vitro–produced chimeric molecule that contained edited sequence at this position (*12*). The gRNAs used in these experiments (gND7FS+2 and gND7FS+4) (Fig. 6A) (*5*) were modified versions of the *C. fasciculata* gRNA that directs the insertion of a single U at this site (*13*). These modifications predict the formation of a more stable anchor duplex with ND7 substrate RNA than could be achieved with the wild-type *C. fasciculata* gRNA and should direct the insertion of two or four uridylates, respectively, at ES1-Cf (Fig. 6A).

Incubation of a 3′-labeled substrate RNA derived from the sequence of *C. fasciculata* ND7 pre-mRNA (ND7FS/TAG) (Fig. 6A) (*5*) with either of these two gRNAs in *T. brucei* mitochondrial extract resulted in the formation of high molecular weight species, but not edited RNA (Fig. 6, B and C). RNase sequencing of these higher molecular weight species indicated that they were gRNA/substrate RNA chimeras with 5′ gRNA sequences linked to the substrate RNA at various sites (9), similar to the chimeras produced in extracts of *C. fasciculata* (*12*). No 3′ cleavage fragments corresponding to gRNA-specified cleavage at ES1-Cf were detected, but multiple products corresponding to cleavage of the substrate RNA 5′ to ES1-Cf were produced and probably recruited into these chimeras.

The region 5′ to ES1-Cf in ND7FS/TAG lacks an extended purine-rich sequence that is found in the corresponding region of the synthetic A6 substrate RNA (dotted lines in Fig. 6A) and is present in most pre-mRNAs that undergo RNA editing (*14*). Its absence may hinder interactions between the gRNA and substrate RNA that allow efficient editing and consequently favor the formation of chimeras over edited product in vitro (*4*).

Therefore, a substrate RNA was constructed in which the region 5′ to ES1-Cf in ND7FS/TAG was replaced with the purine-rich upstream sequence found in A6-eES1 [to produce A6-ND7FS/TAG (Fig. 6A)] (*5*). Incubation of 3′ end-labeled A6-ND7FS/TAG with mitochondrial extract and either of the two gRNAs resulted in the formation of a gRNA-directed 3′ cleavage product, chimera-sized RNAs, as well as RNA species with the size expected for edited product (Fig. 6, B and C). RNA sequence analysis of edited product-sized molecules isolated from preparative reactions confirmed that the predicted number of U's were inserted into ES1-Cf in both instances (9). Thus, the 5′ sequence containing the extended stretch of purines found in the A6-eES1 construct somehow confers the ability to edit this substrate RNA.

Less abundant RNAs that are slightly larger than the edited RNA are also produced, and sequence analysis of one of these (Fig. 6C) showed that three U's were inserted into ES1-Cf (9). These molecules may represent the in vitro analogs of partially edited mitochondrial RNAs that contain numbers of U's at editing sites that differ from the number in the mature mRNA (*15–17*).

Formation of edited A6-ND7FS/TAG RNA was dependent on gRNA, mitochondrial extract, UTP, and ATP (Fig. 6C), as was the case with gRNA-specified U insertion into an A6 substrate RNA [Fig. 2 (9)]. As was also observed with the A6 substrate RNA [Fig. 2 (9)], cleavage of the substrate RNA at the editing site did not require exogenous UTP or ATP, but did require gRNA and mitochondrial extract (Fig. 6B).

Chimeras as end products. Chimeric gRNA/pre-mRNA molecules have been proposed to be intermediates in models of RNA editing that involve either transesterification (*3*) or a modified version of the cleavage and ligation reaction pathway (*8*). In both models, the 3′ oligo(U) "tail" of the gRNA is proposed as the source of the U's that are inserted in the pre-mRNA. During in vitro U insertion, however, the presence of the oligo(U) tail of the gRNA was not sufficient to promote formation of edited product (Figs. 2B and 6C). Rather, U insertion was dependent on the presence of UTP (Figs. 2, 4, 5, and 6), which donates UMP to the 3′ end of the 5′ cleavage product and is incorporated into edited RNA (Fig. 4). These data support models that implicate

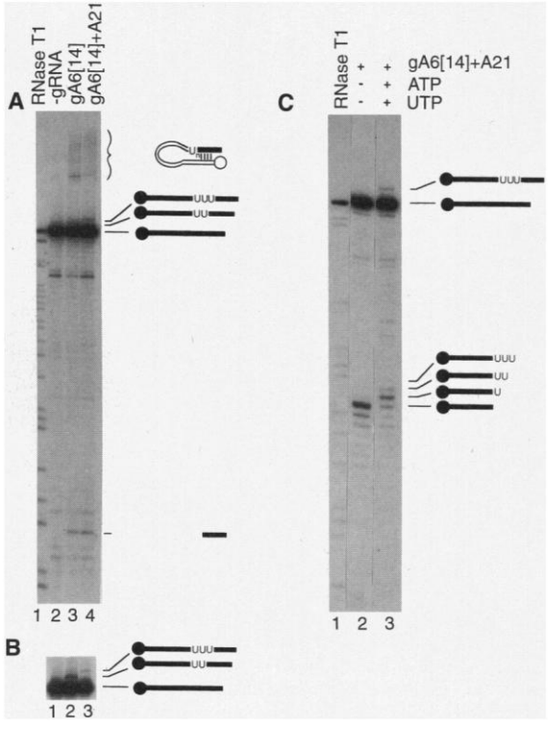

Fig. 5. gRNAs specify the number of inserted uridylates. (**A**) Electrophoresis of 3′ end-labeled A6-eES1 substrate RNA as in Fig. 2A. The RNA was either partially digested with RNase T1 (lane 1) or used in in vitro processing reactions (7) that lacked gRNA (lane 2) or contained gA6[14] (lane 3) or gA6[14]+A21 (lane 4). (**B**) Extended electrophoresis of samples from (A) to enhance resolution of substrate RNA and edited product. Lanes 1, 2, and 3 show samples from lanes 2, 3, and 4 in (A), respectively. (**C**) Addition of nucleotides to the 5′ cleavage product directed by the mutant gRNA. Substrate A6-eES1 RNA, prepared as in Fig. 4A, after partial digestion with RNase T1 (lane 1) or used in in vitro reactions with gA6[14]+A21 (lanes 2 and 3) and either without (lane 2) or with (lane 3) ATP and UTP.

free UTP as the source of the inserted U's (*2*, *4*) and disfavor models which propose that gRNAs donate U's through chimeric intermediates.

If free UTP is required in a reaction pathway that utilizes chimeric intermediates, then chimeras should appear before edited RNA. However, in vitro time course experiments with 3′ end-labeled A6-eES1 substrate RNA showed that chimeras appeared subsequent to edited RNA (Fig. 7). In contrast, the 3′ cleavage product appeared slightly before both edited RNA and chimeras and reached a steady-state level, whereas the level of edited RNA and chimeras continued to increase. Therefore, both edited product and chimeras accumulated with the characteristics of in vitro end products that utilized the 3′ cleavage product as an intermediate in their formation.

We recognize that chimeras could be short-lived intermediates required in the formation of edited RNA and only accumulate at later incubation times as a result of partial loss of functionality of the editing machinery. Given the sum of our data, however, we think it more likely that, as previously suggested for the U-deletion reaction (*4*), chimeras result from an aberrant editing reaction that occurs at the expense of the formation of edited RNA (see below).

A model for U-insertion editing. We have demonstrated gRNA-specified U insertion with two different pre-mRNA/gRNA pairs. Our studies indicate that in vitro U insertion proceeds by a mechanism similar to that of in vitro U deletion (Fig. 8), both of which are close to the original proposal for RNA editing (*2*). Both reactions appear to require RNA endonuclease, an activity that either adds or removes U's from the 3′ end of RNAs, and RNA ligase—all of which are present in the mitochondria of kinetoplastid protozoa (*18*, *19*). In *T. brucei*, each of these activities sediments in a region of glycerol gradients that contains endogenous gRNAs and pre-mRNAs (*20*, *21*) and which partially overlaps with the region that contains in vitro U-deletion activity (*21*), providing strong circumstantial evidence for their involvement in RNA editing.

The first step during both U insertion (Figs. 2A, 5A, 6B, and 7) and U deletion (*4*) appears to be endonucleolytic cleavage of the substrate RNA at the site of gRNA/substrate RNA mismatch immediately 5′ to the gRNA/substrate RNA duplex. Thus, gRNAs seem to determine the site to be edited by directing endonucleolytic cleavage. gA6[14]-directed cleavage at ES1 of the A6 substrate RNA occurs when this site contains unedited sequence (*4*), but not edited sequence (Fig. 2), suggesting that ES1 is protected from cleavage by editing (*16*).

Cleavage of the substrate RNA at the editing site is dependent on gRNA during the U-insertion reaction, but it is also cleaved at other sites in a gRNA-independent manner (Figs. 2A, 4A, 5A, and 6B). These latter cleavage events may be carried out by the same endonuclease that promotes gRNA-

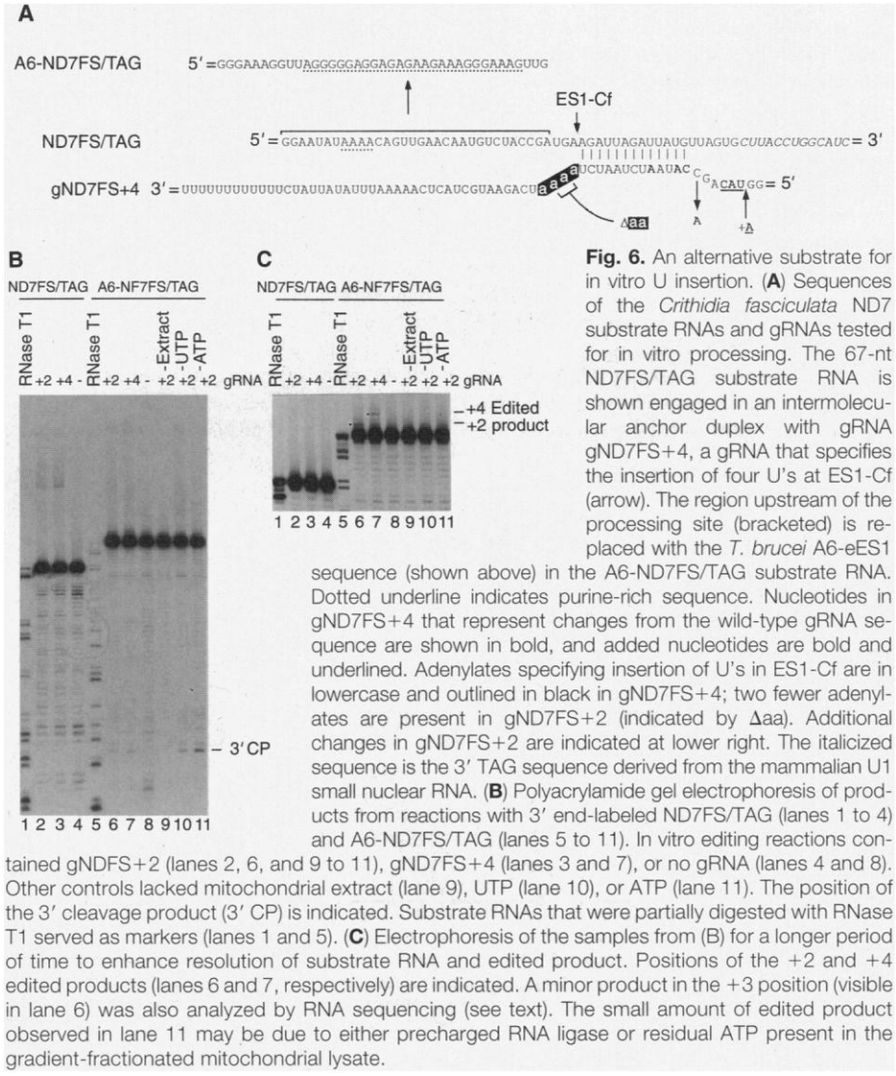

Fig. 6. An alternative substrate for in vitro U insertion. (**A**) Sequences of the *Crithidia fasciculata* ND7 substrate RNAs and gRNAs tested for in vitro processing. The 67-nt ND7FS/TAG substrate RNA is shown engaged in an intermolecular anchor duplex with gRNA gND7FS+4, a gRNA that specifies the insertion of four U's at ES1-Cf (arrow). The region upstream of the processing site (bracketed) is replaced with the *T. brucei* A6-eES1 sequence (shown above) in the A6-ND7FS/TAG substrate RNA. Dotted underline indicates purine-rich sequence. Nucleotides in gND7FS+4 that represent changes from the wild-type gRNA sequence are shown in bold, and added nucleotides are bold and underlined. Adenylates specifying insertion of U's in ES1-Cf are in lowercase and outlined in black in gND7FS+4; two fewer adenylates are present in gND7FS+2 (indicated by Δaa). Additional changes in gND7FS+2 are indicated at lower right. The italicized sequence is the 3′ TAG sequence derived from the mammalian U1 small nuclear RNA. (**B**) Polyacrylamide gel electrophoresis of products from reactions with 3′ end-labeled ND7FS/TAG (lanes 1 to 4) and A6-ND7FS/TAG (lanes 5 to 11). In vitro editing reactions contained gNDFS+2 (lanes 2, 6, and 9 to 11), gND7FS+4 (lanes 3 and 7), or no gRNA (lanes 4 and 8). Other controls lacked mitochondrial extract (lane 9), UTP (lane 10), or ATP (lane 11). The position of the 3′ cleavage product (3′ CP) is indicated. Substrate RNAs that were partially digested with RNase T1 served as markers (lanes 1 and 5). (**C**) Electrophoresis of the samples from (B) for a longer period of time to enhance resolution of substrate RNA and edited product. Positions of the +2 and +4 edited products (lanes 6 and 7, respectively) are indicated. A minor product in the +3 position (visible in lane 6) was also analyzed by RNA sequencing (see text). The small amount of edited product observed in lane 11 may be due to either precharged RNA ligase or residual ATP present in the gradient-fractionated mitochondrial lysate.

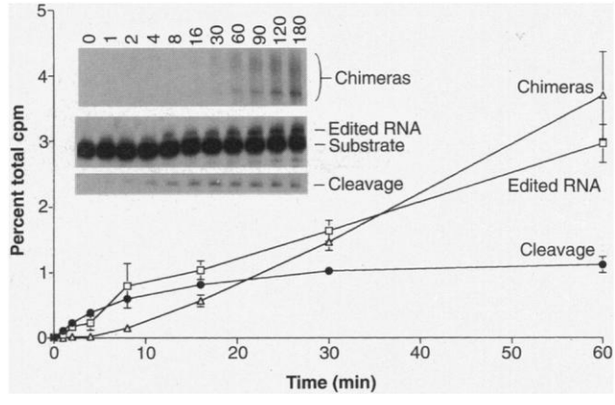

Fig. 7. Time course of processing. Samples from a scaled-up reaction like that shown in Fig. 2A, lane 2, were collected at the indicated times and reaction products fractionated by polyacrylamide gel electrophoresis (**inset**). Products labeled cleavage, substrate, edited RNA, and chimeras were excised and their activity was determined by liquid scintillation counting. The graph shows the relative levels of 3′ cleavage product, edited RNA, and chimeras formed over the first 60 min. The average values from two independent experiments are expressed as percentage of the counts per minute (cpm) of each species relative to the total activity in each lane, and the error bars indicate the range of the two values. The range in values for some time points was too small to be shown.

dependent cleavage and may take place in the loop of an intramolecular stem-loop structure (*11*, *19*). These structures could mimic the gRNA/substrate RNA anchor duplex and target the substrate RNA for cleavage, possibly resulting in gRNA-independent U insertion similar to that observed in mitochondrial extracts of *Leishmania tarentolae* (*22*). Although further work is needed to identify the RNA endonuclease and determine its precise substrate recognition characteristics, substrate RNA cleavage during both the insertion and deletion reaction is directed by gRNA and produces a 3′-half RNA that terminates in a 5′ monophosphate (Figs. 2A and 3C) and a 5′-half RNA that terminates in a 3′ hydroxyl (Fig. 4A) [(*4*), and references therein].

The location of substrate RNA cleavage during in vitro U insertion and U deletion suggests that editing sites are selected by a gRNA in a strict 3′ to 5′ order during multiple cycles of editing. However, many partially edited pre-mRNAs found in vivo do not display a strict 3′ to 5′ polarity of editing. Instead, partially edited molecules often have a junction region that contains edited sites 5′ to unedited ones, or a U content at editing sites and nonediting sites that matches neither the gene nor the mature mRNA (*15*–*17*), or both. How can these partially edited molecules be reconciled with a strict 3′ to 5′ choice of editing sites suggested by the location of substrate RNA cleavage observed in this study? Among the various possibilities, pre-mRNAs may be processed in vivo with noncognate gRNAs (*23*, *24*) or with cognate gRNAs that specify a sequence intermediate between that of the gene and the mature mRNA (which then requires further processing by a different gRNA to reach the mature sequence) (*25*). Alternatively, metastable intermolecular associations between gRNA and pre-mRNA may create duplexes that direct pre-mRNA cleavage, and hence processing, at noncontiguous sites (*17*, *24*).

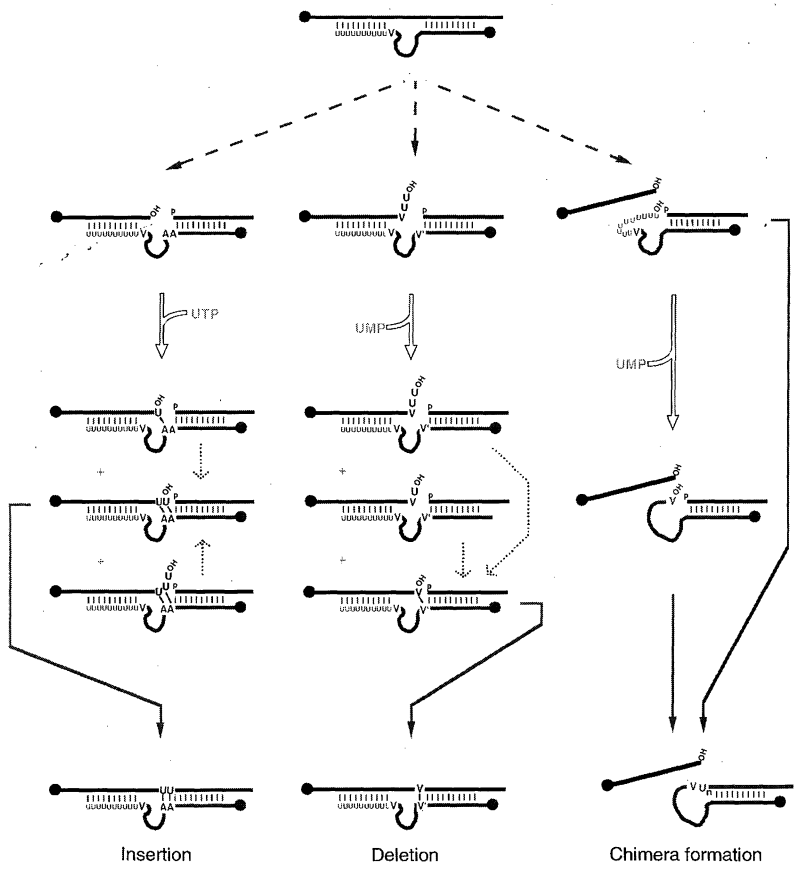

Fig. 8. A model for RNA editing in kinetoplastids. Schematic showing models for U insertion (left), U deletion (center), and chimera formation (right). All reaction pathways begin with gRNA-directed endonucleolytic cleavage next to the anchor duplex (dashed arrows). U addition or removal from the 3′ end of the 5′ cleavage product (left and center) or the 3′ end of the gRNA (right, open arrows) could be catalyzed by TUTase and produce a population of 5′ cleavage intermediates that differ in the number of U's at their 3′ ends. RNA ligation (solid arrows) preferentially joins a correctly processed 5′ cleavage intermediate with a 3′ cleavage intermediate during U insertion and deletion (left and center, respectively) or joins the (processed) gRNA with the 3′ cleavage intermediate during chimera formation (right). The appropriate 5′ cleavage intermediate (left and center) may be selected by base-pairing with the gRNA sequence across from the processing site. 5′ cleavage products with an inappropriate number of U's may be further processed to the correct number (dotted arrows). V, a non-U nucleotide.

TUTase is thought to play a role in RNA editing because it is found in the mitochondria of organisms undergoing U-insertion or U-deletion RNA editing (*24*) and because it cosediments with mitochondrial pre-mRNAs and gRNAs. TUTase uses UTP to donate 5′-UMP to the 3′ end of RNAs, just as U's derived from free UTP are added to the 3′ hydroxyl of the 5′ cleavage product during the U-insertion reaction (Fig. 8, left). TUTase may also catalyze the reverse reaction and remove 5′-UMP from the 3′ end of the 5′ cleavage product during the U-deletion reaction (Fig. 8, center) (*4*); alternatively, this activity could be performed by a distinct 3′ exonuclease specific for U residues (*2*).

Four lines of evidence implicate an RNA ligase in RNA editing. (i) RNA ligase activity cosediments with in vitro editing activity (*21*). (ii) The cleavage products generated during both U insertion and U deletion have termini that can be acted upon by RNA ligase. (iii) U deletion is dependent on hydrolysis of the α-β phosphate bond of ATP (*10*), as is mitochondrial RNA ligase function (*26*). (iv) RNA ligase is responsible for in vitro formation of gRNA/pre-mRNA chimeric molecules (*26*), which are probably formed by the same machinery that catalyzes the production of edited RNA.

The number of inserted U's, and therefore the edited sequence, must be determined at the RNA ligation step because the gRNA sequence at the processing site does not seem to determine the number of U's added to the 3′ end of the 5′ cleavage product {compare the number of U's added to this product in reactions with wild-type gRNA or gA6[14]+A21 (Fig. 4A, lane 4, and Fig. 5C, lane 3, respectively)}. Specificity during ligation may be supplied by gRNA base pairing–mediated juxtaposition of the 3′ cleavage product with the correctly processed 5′ cleavage product (Fig. 8). Thus, gRNA may act as a "splint," analogous to a DNA oligonucleotide during RNA ligation reactions catalyzed by T4 DNA ligase (*27*). Specificity during the ligation step is obviously not absolute because edited RNAs that contain a number of U's are produced at a low frequency, both greater and less than that specified by the gRNA (Fig. 6).

As demonstrated by the two different ND7 substrate RNAs, edited RNA is detectable only when there is substantial potential for base pairing between the 3′ region of the gRNA and the substrate RNA sequence 5′ to the editing site (Fig. 6). Similar results from studies of in vitro U

deletion led to the proposal that chimera formation results when the 3′ end of the gRNA occupies the position held by the 3′ end of the 5′ cleavage product during the productive editing pathway (Fig. 8, right) (*4*). Thus, chimeras may be aberrant end products, and not intermediates, of editing.

This model for chimera formation predicts that the number of U's linking gRNA and substrate RNA in at least a subset of chimeras should be determined by the gRNA sequence at the processing site. The $D_{n=2}$ chimeras (Fig. 2) fulfill this expectation in that two U's are present at the linkage site, just as two U's are specified by gA6[14]. Chimeras formed during in vitro U deletion (*4*) and in other in vitro studies (*12*) also show these characteristics. $D_{n>2}$ chimeras may also fulfill this criterion because they could result from transient interactions in which two U's at the 3′ end of the gRNA form base pairs with the two A's in the gRNA at the editing site.

However, as evidenced by the production of $D_{n=0}$ chimeras (Fig. 2A, lane 4), chimera formation in vitro does not seem to require precise alignment of the 3′ end of the (processed) gRNA and the 5′ end of the 3′ substrate RNA cleavage product. This lack of precise alignment seems also to be applicable for chimeras produced in vivo, because some of these lack U's at the point of linkage (*28*). Reverse TUTase or a 3′ exoribonuclease activity may process the 3′ end of gRNAs before in vitro formation of $D_{n=0}$ and (possibly) $D_{n=2}$ chimeras, and act similarly in vivo.

Our findings provide the first demonstration of gRNA-specified in vitro U insertion and therefore display the characteristic defining authentic kinetoplastid U-insertion editing. Previous reports of in vitro U insertion were either gRNA-independent (*22*) or resulted in products with a number of U's that differ from that specified by the gRNA (*29*). On the basis of studies in which our in vitro system was used to probe the mechanism of U insertion, we conclude that gRNA performs at least two functions during processing: selection of a position for endoribonucleolytic cleavage and preferential selection of molecules for RNA ligation. We also conclude that 5′ and 3′ substrate RNA cleavage products are editing intermediates during both the U-insertion and the U-deletion reaction, whereas chimeras are nonproductive end products, rather than intermediates, of RNA editing.

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5. The cloned edited product of U deletion at ES1 (*10*) was used as a PCR template in a reaction containing oligonucleotides A6short (*4*) and A6U2/3′BamH1 (5′-GGATCCGATGCCAGGTAAGTATTCTATAACTCCAATCACAAC-3′). The PCR product was gel-purified and reamplified with oligonucleotides A6short and A6RT2 (*10*) to produce a T7 polymerase transcription template for A6-eES1. gA6[14] was synthesized as described (*6*). The transcription template for gA6[14]+A21 was produced by Dra I digestion of the product resulting from PCR of gA6[14]NX (*30*) with the mutagenic oligonucleotide T7gA6ES2+3 (5′-GTAATACGACTCACTATAGGATATACTATAACTCCGATAAACGAATCAG-3′) and -21PUC118 (5′-TGTAAAACGACGGCCAGT-3′). A DNA fragment (ND7FS) containing sequence of the *C. fasciculata* ND7 gene (frameshift region) (*13*) was constructed by annealing the oligonucleotides ND7FS5′ (5′-GGCGGAATTCGTAATACGACTCACTATAGGAATATAAAACAGTTGAACAATGTCTAC-3′) and ND7FS3′ (5′-CGCGTCTAGACACTAACATAATCTAATCTTCATCGGTAGACATTGTTCAACTGT-3′) and subsequent fill-in of the 5′-protruding ends with T4 DNA polymerase (New England Biolabs). A template for transcription of ND7FS/TAG was then generated by mutagenic PCR with the oligonucleotides Eco RI/T7 (5′-CGGCGGAATTCTGTAATACGACTCAC-3′) and ND7FS3′/2 (5′-GATGCCAGGTAAGCACTAACATAATCTAATC-3′) with ND7FS as template DNA. The gRNA gND7FS+2 was prepared by in vitro transcription with T7 RNA polymerase (Ambion) from a DNA template generated by annealing the oligonucleotides 5′gND7FS+2 (5′-GCGGAATTCGTAATACGACTCACTATAGGATACAGACATAATCTAATCTAATCAG-3′) and 3′gND7FS+2 (5′-AAAAAAAAAAAAGATAATATAAATTTTTGAGTAGCATTCTGATTAGATTAGAT-3′) and subsequent fill-in with T4 DNA polymerase. gND7FS+4 was constructed in the same way with the oligonucleotides 5′gND7FS+4 (5′-GGCGGAATTCGTAATACGACTCACTATAGGTACAGCCATAATCTAATCTAAAATCAG-3′) and 3′gND7FS+4 (5′-AAAAAAAAAAAAAAAGATAATATAAATTTTTGAGTAGCATTCTGATTTTAGATTAG-3′). The template for in vitro transcription of the hybrid substrate RNA A6-ND7FS/TAG was synthesized by mutagenic PCR with the oligonucleotides Eco RI/T7 and A6-CfND7/5′ (5′-GATGCCAGGTAAGCACTAACATAATCTAATCTTCACAACTTTCCCTTTC-3′) and with a PCR product encoding A6short/TAG.1 (*4*) as DNA template. Products of T7 polymerase transcription were purified on 10% polyacrylamide, 7 M urea, 1× tris-borate EDTA gels. A6-eES1, ND7FS/TAG, and A6-ND7FS/TAG were radiolabeled at their 3′ ends with [5′-^{32}P]pCp, and A6-eES1 was radiolabeled at its 5′ end with [α-^{32}P]GTP as described (*4*) and purified on 9% polyacrylamide, 7 M urea, 1× tris-borate EDTA gels. We have consistently observed that ND7FS/TAG runs as a doublet even after gel purification. The terminal 2′ and 3′ hydroxyls of A6-eES1 were oxidized by sodium-*m*-periodate treatment (*4*), which was >99% efficient at blocking the 3′ end as judged by subsequent [5′-^{32}P]pCp labeling.
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7. In vitro reactions were performed under conditions similar to the those previously described (*4*). Reactions (30 μl) were incubated for 1 hour at 28°C and contained 10 μl (~10 μg of protein) of a 20 to 35S glycerol gradient fraction of *T. brucei* mitochondrial lysate (*4*), 50 μM UTP, 0.25 pmol of editing substrate, 2.5 pmol of gRNA, 37 mM KCl, 10 mM MgOAc, 25 mM tris-HCl (pH 8.0), 3 mM ATP, 0.5 mM dithiothreitol, 1 mM EDTA, 5 mM $CaCl_2$, and 1 μg of *Torula* yeast RNA. Reactions were stopped by the addition of 70 μl of NET-2 [150 mM NaCl, 50 mM tris-HCl (pH 7.5), and 0.05% Nonidet P-40], and 1 μg of glycogen was added. RNA was collected by extraction with phenol and precipitation with ethanol, and samples were run on 9% polyacrylamide, 7 M urea, 1× tris-borate EDTA gels, which were dried and exposed to film. End-labeled RNAs were subjected to partial digestion by RNase T1 (Boehringer Mannheim) to serve as size markers. RNase T1 cleaves 3′ of guanylate residues, leaving a 3′ phosphate. Partial RNase T1 digestions were carried out for 15 min at 55°C and contained 0.25 pmol of end-labeled RNA, 0.3 U of RNase T1, 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 4.2 M urea, *Torula* yeast RNA (0.6 μg/μl), 0.02% bromophenol blue, and 0.02% xylene cyanole. Product A was purified from a 10× reaction like that shown in Fig. 2A, lane 2, reverse-transcribed with Superscript II (Gibco-BRL) with 5′ kinased oligonucleotide A6-RT (*6*) as a primer, and digested with RNase A. The size of the primer extension product was compared to that of reverse transcription sequencing (*10*) of A6-eES1 with the same primer. In Fig. 3C, 4 μM [α-^{32}P]UTP and unlabeled, periodate-treated A6-eES1 were substituted for unlabeled UTP and for end-labeled A6-eES1, respectively, and reactions were scaled up twofold. After in vitro reaction and electrophoresis, RNAs with the mobility expected of edited product were excised, eluted, and precipitated with ethanol. One-half of the gel-purified samples was treated with RNase H (Gibco-BRL) in the presence of 1.6 μg of oligonucleotide anti-gA6anchor (5′-GATCTTATTCTATAACTCCAA-3′) as directed by the manufacturer. Complete RNase T1 digestion was performed at 37°C for 15 min and included 20 U of RNase T1, 50 mM tris-HCl (pH 7.4), and 2 mM EDTA.
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