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Direct Visualization of Uridylate Deletion In Vitro Suggests a Mechanism for Kinetoplastid RNA Editing

Scott D. Seiwert,*‡ Stefan Heidmann,*‡ and Kenneth Stuart*†
*Seattle Biomedical Research Institute
4 Nickerson Street
Seattle, Washington 98109
†Department of Pathobiology
University of Washington
Seattle, Washington 98195

Summary

Deletion of uridylates from the 3′-most editing site of synthetic ATPase 6 pre-mRNA can be visualized directly by coincubation of a radiolabeled substrate RNA and a synthetic gRNA in 20S fractions of T. brucei mitochondrial lysates. Substrate RNA cleavage is gRNA directed and occurs 3′ to the uridylates to be deleted. U residues appear to be sequentially removed from the 3′ end of the 5′ cleavage product prior to religation of the two pre-mRNA halves. gRNA/mRNA chimeric molecules are also produced. Time course experiments indicate that chimeras appear after cleavage intermediates and edited product. Furthermore, a mutant gRNA promotes formation of edited product but not detectable chimeras. Our results suggest a model for kinetoplastid RNA editing in which chimeric molecules are nonproductive end products of editing and not intermediates that serve as a repository for deleted U's.

Introduction

In the mitochondria of kinetoplastid protozoa, uridylate (U) residues are inserted and deleted within the coding regions of pre-messenger-RNAs (pre-mRNAs) by an unusual form of RNA processing referred to as RNA editing (Benne, 1994; Seiwert, 1995; Simpson and Thiemann, 1995). RNA editing requires small trans-acting RNAs (guide RNAs or gRNAs) that are complementary to portions of edited sequences (Blum et al., 1990). The function(s) of gRNAs in RNA editing can be inferred from their tripartite primary structure. gRNAs have an "anchor" sequence of 4–14 nucleotides at their 5′ end, an internal "information" sequence, and a nonencoded 3′ oligo(U) "tail" of 5–20 residues. During editing, the anchor sequence of a gRNA forms a short intermolecular duplex with its cognate pre-mRNA immediately downstream of the site(s) requiring U insertion or deletion. Bulged purines in the informational portion of the gRNA then direct U insertion while U's bulged in the pre-mRNA are deleted. Three models have been proposed for the mechanism of U insertion and deletion (Blum et al., 1990, 1991; Cech, 1991; Harris and Hajduk, 1992); central to each is the role of the oligo(U) tail of the gRNA.

The original model for kinetoplastid mitochondrial RNA editing suggested that free uridine triphosphate (UTP) in the mitochondrion serves as the source of U's inserted into pre-mRNAs (Blum et al., 1990). Editing is initiated when an endonuclease cleaves the pre-mRNA across from a purine bulged in the gRNA (in the case of U addition) or cleaves 3′ to a U bulged in the pre-mRNA (for U deletion). Terminal uridylyltransferase (TUTase) then adds or deletes uridine monophosphate(s) to/from the 3′ end of the 5′ cleavage product as specified by the gRNA sequence at the processing site. After the correct sequence is achieved, the two half RNAs are ligated to form a product edited at the specified internucleotide site. In this model, the 3′ oligo(U) tail of the gRNA may interact with purine-rich sequence upstream of the processing site (Blum and Simpson, 1990).

The two remaining models for editing suggest the oligo(U) tail of the gRNA plays a direct role in the chemical steps of the reaction by serving as a reservoir for the inserted and deleted U's (Blum et al., 1991; Cech, 1991; Harris and Hajduk, 1992). In one of these models, endonucleolytic cleavage of the substrate RNA generates 5′ and 3′ half molecules as in the preceding model (Harris and Hajduk, 1992). However, instead of U addition or deletion to/from the 3′ end of the 5′ cleavage product, the oligo(U) tail of the gRNA is ligated to the 3′ cleavage product to generate a gRNA/mRNA chimera. If one or more U's are to be added, purines in the gRNA's informational section pair to U's derived from the oligo(U) tail of the same molecule and the chimera is cleaved 5′ to the original 3′ end of the gRNA. In this case, extra U's would be donated from the gRNA to the 5′ end of the 3′ cleavage product. If U's are to be deleted, cleavage of the chimera would occur 3′ to a U derived from the initial 3′ cleavage product, and consequently these residues would be donated to the tail of the gRNA. Subsequent ligation of the two half RNAs would then produce an edited product.

Edited molecules could also be generated in a reaction pathway consisting of two sequential transesterifications (Blum et al., 1991; Cech, 1991), similar to those of RNA self-splicing reactions. In this model, pre-mRNA scission at the editing site is coupled to chimera formation in a phospho-transfer reaction in which the 3′ OH of the gRNA acts as the attacking nucleophile. Pseudo-reversal of this reaction with the 3′ OH of the 5′ cleavage product acting as a nucleophile would insert U('s) at the processing site if attack occurred 5′ of the original gRNA terminus or delete U('s) if attack occurred 3′ to the original terminus.

Despite these detailed models for the role of gRNAs in RNA editing and the nature of the chemical steps of U insertion and deletion (Blum et al., 1990, 1991; Cech, 1991; Harris and Hajduk, 1992) there is a paucity of information directly bearing on the mechanism of these reactions. Pre-edited RNA–specific endonuclease, TUTase, and RNA ligase activities have been detected in the mitochondria of kinetoplastids (White and Borst, 1987; Bakalara et al., 1989; Harris et al., 1992, Simpson et al., 1992). In Trypanosoma brucei, these activities coexist in complexes of 20S and 35–40S (Pollard et al.,

‡These authors contributed equally to this work.

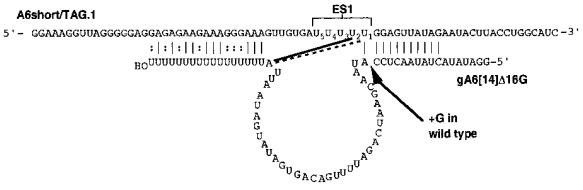

Figure 1. Schematic of Editing Substrate RNA and gRNAs

A6short/TAG.1 (73 nucleotides in length) represents a shortened version of the A6/TAG.1 substrate RNA used in our previous studies (Seiwert and Stuart, 1994). Editing site 1 (ES1) is indicated by a bracket. gA6[14]Δ16G (70 nucleotides in length) is shown basepaired to A6short/TAG.1 by the anchor duplex and by a duplex between the oligo(U) tail and a purine-rich region found upstream of processing sites. Watson–Crick base pairs are indicated by lines, while G:U base pairs are denoted by colons. The long solid and dashed lines indicate the gRNA/pre-mRNA linkage sites in D-type chimeras in reactions using wild-type gA6[14] and gA6[14]Δ16G, respectively (see Figure 2B and text). The position of the additional guanylate residue in wild-type gA6[14] is marked by an arrow.

1992; Piller et al., 1995b; Sabatini and Hajduk, 1995). These findings, and the recent implication of an RNA ligase in editing (Rusché et al., 1995; Sabatini and Hajduk, 1995), favor those models in which catalysis is performed by protein enzymes. The existence of chimeric molecules in vivo (Blum et al., 1991; Read et al., 1992; Arts et al., 1993) and their production in vitro (Blum and Simpson, 1992; Harris and Hajduk, 1992; Koslowsky et al., 1992) have been taken as strong evidence for models in which the gRNAs' U tail serves as a reservoir for U's. However, it has not been demonstrated that chimeras represent true reaction intermediates. The in vitro U deletion system we previously reported demonstrated the transfer of genetic information from gRNA to pre-mRNA using an assay that indirectly monitored processing (Seiwert and Stuart, 1994). To investigate the mechanism of this reaction, we wished to track the processing of the bulk population of substrate RNA in order to identify possible intermediates and probe the biochemistry of the reaction.

Results

To facilitate the direct analysis of in vitro reaction products, we have used an editing substrate based on the pre-mRNA for ATPase 6 that is only 73 nucleotides long and contains five U's within editing site 1 (ES1) (A6short/TAG.1; Figure 1). This pre-mRNA is identical to that used in our previous studies (Seiwert and Stuart, 1994), except it has 41 nucleotides upstream of ES1, rather than 127 nucleotides. Di-deoxynucleotide terminated primer extension analysis (Seiwert and Stuart, 1994) shows that this shortened substrate RNA is processed to the same extent in vitro as the longer A6/TAG and A6/TAG.1 pre-mRNAs (data not shown).

Direct Visualization of Processing

In our previous work, we have shown that the number of U's deleted from an editing substrate RNA is programmed by the gRNA sequence that basepairs across the editing site (Seiwert and Stuart, 1994) and that deletion is carried out by a particle that sediments at ~20S in glycerol gradients (R. A. Corell et al., 1996). To determine if processing can be visualized directly, 3′ end–labeled substrate RNA was incubated in ~20S glycerol gradient fractions under the conditions described in Experimental Procedures either with wild-type gA6[14] (which directs the deletion of 3 U's from ES1), or with gA6[14]Δ16G (which directs the deletion of 4 U's from ES1), or without gRNA (Figure 2A, lanes 3, 4, and 5, respectively). In confirmation of our earlier work (Seiwert and Stuart, 1994), no species with the mobility of edited product is produced without gRNA (lane 5). Besides the input substrate RNA (labeled C in Figure 2A), four species are produced in reactions that include gRNA (labeled A, B, D, and E). In both cases, product B has the size expected for edited product. To determine the sequence of B-type molecules generated in reactions with wild-type gRNA (lane 3) and gA6[14]Δ16G (lane 4), they were purified from preparative reactions and subjected to partial digestion with either RNase T1 (Figure 2B, lanes 1 and 4), Bacillus cereus RNase (Figure 2B, lanes 2 and 5), or RNase U2 (Figure 2B, lanes 3 and 6). B-type molecules from reactions that included wild-type gRNA contain 2 U's at ES1 (lane 2), while B from reactions in which gA6[14]Δ16G is used contains 1 U at ES1 (lane 5), as is predicted by the sequence of the respective gRNAs (see Figure 1). Examination of the sequence of both edited products indicates that they are identical to the input pre-mRNA at all other positions (lanes 1–6). Thus, ES1 is specifically targeted for processing by gRNA, in agreement with our earlier findings (Seiwert and Stuart, 1994).

In reactions that include gRNA, a cleavage product representing the 3′ half of substrate RNA is also detected (labeled A in Figure 2A). The identity of this species has been confirmed by partial digestion with RNase T1 of material purified from preparative reactions (data not shown). The size of product A is determined by the sequence of the gRNA used (compare A in Figure 2A, lanes 3 and 4). The pre-mRNA is also cleaved to a lower extent in this region when gRNA is omitted from the reaction (Figure 2A, lane 5). It has been proposed that, in the absence of gRNA, ES1 of this substrate RNA forms the loop of an intramolecular stem–loop structure (Piller et al., 1995a), raising the possibility that the pre-mRNA alone can adopt a conformation that is susceptible to nuclease attack in the absence of gRNA (see Discussion). Therefore, cleavage at ES1 may not strictly require gRNA, but is nonetheless enhanced and more specifically directed to a single site by gRNA. The site of gRNA-directed cleavage suggests that pre-mRNA scission occurs 3′ of the U's to be deleted (compare the migration of these cleavage products [Figure 2A, lanes 3 and 4] with the migration of partial RNase T1 and hydroxyl ladders of the input substrate RNA [Figure 2A, lanes 1 and 2]). Since partial RNase T1 digestion and hydroxyl cleavage markers carry 5′ hydroxyl groups and a previously identified pre-edited RNA–specific cleavage activity leaves a 5′ monophosphate on 3′ cleavage products (Piller et al., 1995a), we considered that the exact phosphodiester bond targeted for cleavage may not be able to be determined by comigration of cleavage products and marker ladders. To characterize the 5′ termini of A products, they were purified from preparative reactions and subjected to treatment with alkaline phosphatase (which removes 5′ and 3′ phosphates from RNA;

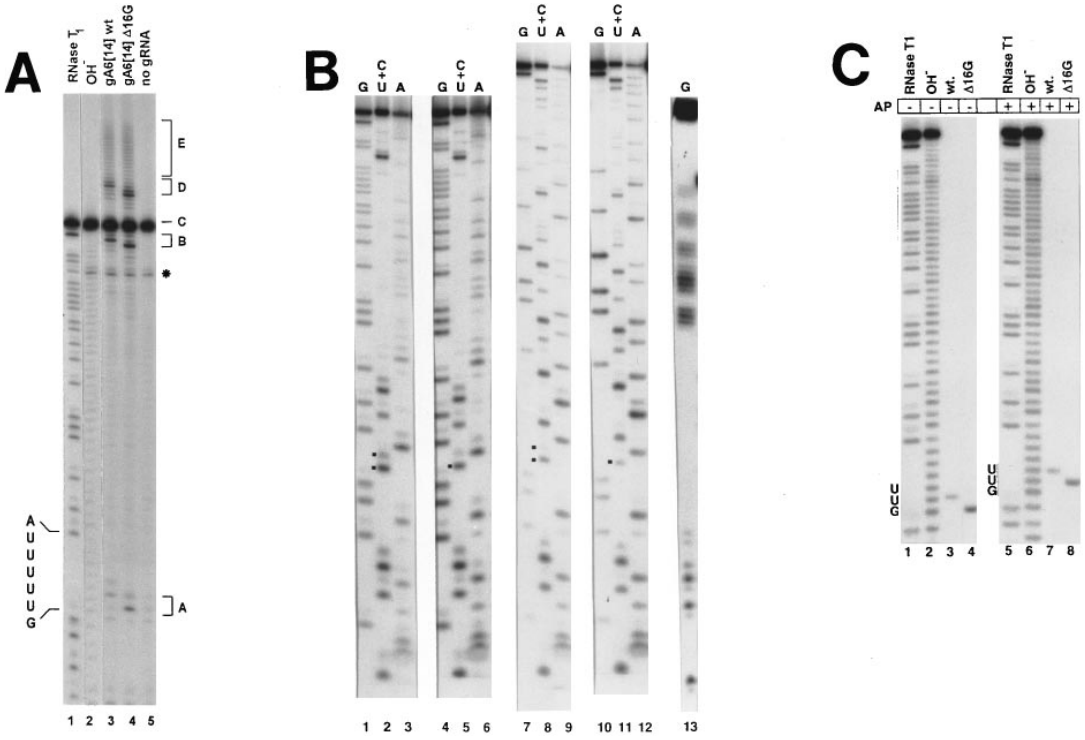

Figure 2. Direct Visualization of Processing Using 3′-End-Labeled Substrate RNA

(A) In vitro processing. Aliquots of 3′ end–labeled substrate RNA were subjected to partial RNase T1 digestion (lane 1) or to partial alkaline hydrolysis (lane 2) to serve as markers, or used in in vitro processing reactions with wild-type gA6[14] (lane 3), with gA6[14]Δ16G (lane 4), or without gRNA (lane 5). Species produced in vitro are designated with letters, and the band produced in an extract-independent fashion and observed in some of our experiments is denoted by an asterisk. The sequence of ES1 is indicated.

(B) Sequence characterization of reaction products. Species labeled B and D were excised from preparative reactions that included wild-type gRNA (lanes 1–3 and 7–9) or gA6[14]Δ16G (lanes 4–6 and 10–12) and subjected to partial digestion with RNase T1 (lanes 1, 4, 7, and 10), B. cereus RNase (lanes 2, 5, 8, and 11), or RNase U2 (lanes 3, 6, 9, and 12). Product E was excised from a preparative reaction that included gA6[14]Δ16G and was subjected to partial RNase T1 digestion (lane 13). Dots in lanes 2 and 5 indicate uridylates at ES1 in the edited product, and dots in lanes 8 and 11 indicate uridylates that link gRNA and pre-mRNA in D-type chimeras.

(C) Determination of the site of gRNA-directed substrate RNA cleavage. Product A was excised from preparative reactions that included wild-type gRNA (lanes 3 and 7) or gA6[14]Δ16G (lanes 4 and 8), and either treated (lanes 7 and 8) or not treated (lanes 3 and 4) with alkaline phosphatase (AP), and electrophoresed next to partial alkaline hydrolysis (lanes 1 and 5) or partial RNase T1 digestion (lanes 2 and 6) ladders of the input RNA that had been treated accordingly.

Figure 2C). Comparison of phosphatase-treated A products (Figure 2C, lanes 7 and 8) and identically treated markers (Figure 2C, lanes 5 and 6) shows that the cleavage products migrate one nucleotide larger in size relative to the markers than when both A products (Figure 2C, lanes 3 and 4) and markers (Figure 2C, lanes 1 and 2) are untreated. This suggests that the 3′ cleavage products generated in vitro carry a 5′ phosphate(s) and that wild-type gRNA promotes cleavage between U3 and U2, while gA6[14]Δ16G promotes cleavage between U2 and U1 (see Figure 1). Runoff primer extension analysis of A products was used to confirm these cleavage sites (data not shown), and the presence of a 5′ monophosphate on A products was directly demonstrated by ligating them to a molecule carrying a 5′ triphosphate and 3′ hydroxyl group (data not shown). Thus, gRNA-directed cleavage occurs at the 3′ terminus (with respect to the gRNA) of the anchor duplex.

RNA sequencing of D-type products generated in reactions using the wild-type gRNA (Figure 2B, lanes 7–9) and gA6[14]Δ16G (Figure 2B, lanes 10–12) indicates that they are gRNA-substrate RNA chimeras linked at ES1. As expected, these chimeras contain pre-mRNA sequence 3′ to ES1, but they lack most of the oligo(U) tail of the gRNA. Digestion with B. cereus RNase shows that two U's link gRNA and pre-mRNA in reactions that included wild-type gRNA (lane 8), while one U links the two RNAs when gA6[14]Δ16G is used (lane 11). Thus, the number of U's linking gRNA and pre-mRNA is determined by the gRNA sequence at the processing site. Since D-type chimeras do not contain enough U's to account for those deleted from the substrate RNA, they cannot serve as a repository for U's, as is proposed for chimeric intermediates (Blum et al., 1991; Cech, 1991; Harris and Hajduk, 1992).

Partial RNase T1 digestion of a pool of several of the bands labeled E in Figure 2A indicates that they are also gRNA/pre-mRNA chimeras (Figure 2B, lane 13). Most are linked at ES1 and contain the same pre-mRNA sequence as the D chimeras characterized in Figure 2B, lanes 7–12. However, the number of nucleotides between the 5′-most G derived from the pre-mRNA and the 3′-most G derived from the gRNA suggests that oligo(U) tails of various lengths link the gRNA and the

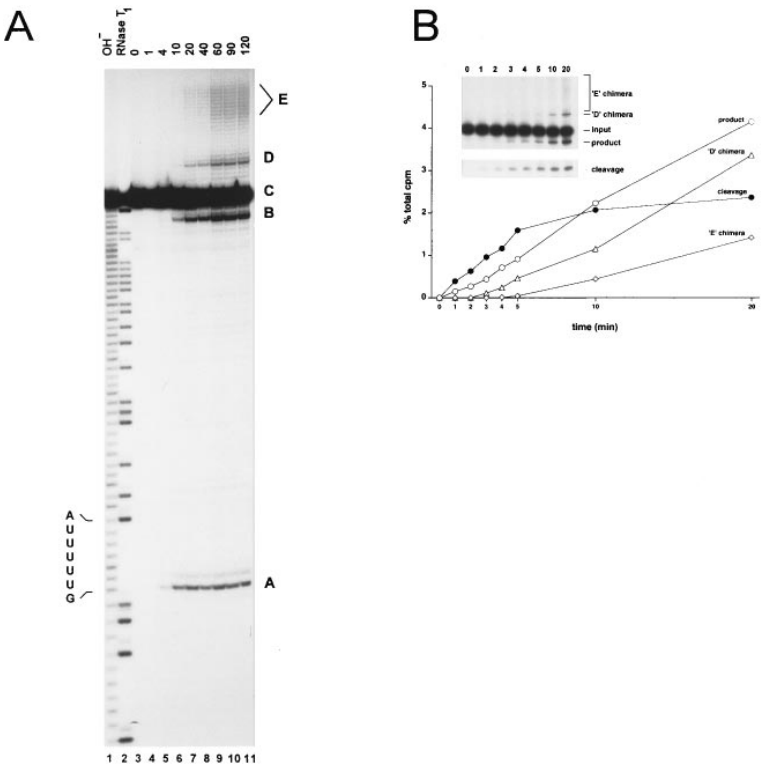

Figure 3. Temporal Relationship of the Appearance of In Vitro Products

(A) Aliquots of 3′ end–labeled RNA were subjected to partial alkaline hydrolysis (lane 1) or to partial RNase T1 digestion (lane 2) to serve as markers, or used in in vitro processing reactions (lanes 3–11) that included gA6[14]Δ16G and were incubated for the indicated times (in minutes). Species produced in vitro are designated as in Figure 2A. The sequence of ES1 is indicated.

(B) Aliquots of 3′ end–labeled RNA were used in in vitro processing reactions like those shown in (A) and were incubated for 1, 2, 3, 4, 5, 10, and 20 min (inset). Species A (3′ cleavage product), B (edited product), C (input RNA), D (D-type chimera), and E (E-type chimera) were excised from the wet gel (inset), counted by liquid scintillation, and the activity of A, B, D, and E was expressed as a fraction of the total cpm collected per lane. Throughout, standard errors in scintillation counting were less than 8% of the measured value.

pre-mRNA, thus these chimeras could serve as a repository for the deleted U's. Chimeras representing gRNAs without an oligo(U) tail linked to various sites upstream of ES1 are sometimes also detected in this region (data not shown).

Temporal Appearance of In Vitro–Produced Species

To investigate the relative appearance of cleavage products, chimeras, and edited product, reactions were carried out as in Figure 2A, lane 4, but aliquots were taken at various times. Figure 3A shows that the 3′ cleavage product is visible after 4 min of incubation, whereas D- and E-type chimeras and edited product become visible after 10 min and accumulate further over time. Thus, in confirmation of our earlier work (Seiwert and Stuart, 1994), edited product appears very early, but unlike the case in crude mitochondrial lysate, it continues to accumulate after 15 min incubation. To resolve the order of appearance of the various products more accurately, a time course experiment examining earlier time points was performed (Figure 3B). In this case, pre-mRNA cleavage can be detected as early as 1 min after the start of the reaction. It clearly precedes formation of both chimeras and edited product, which are roughly coincident with one another (see inset in Figure 3B). Quantitation reveals that the concentration of the 3′ cleavage product reaches a plateau at ~10 min while the amounts of edited product and both types of chimeras increase steadily (Figure 3B). The longer time course (Figure 3A) corroborates these results for time points beyond 20 min. The early appearance of the 3′ cleavage product and its plateau as edited RNA accumulates are consistent with it being an intermediate in the editing reaction. In contrast, D-type and E-type chimeras appear coincident with, or slightly after, edited RNA and accumulate over time, which is inconsistent with a precursor–product relationship to the edited RNA.

U's Are Deleted from the Initial 5′ Cleavage Product

The size of the 3′ cleavage product suggests that the U's to be deleted may be carried on the 3′ end of the 5′ cleavage product. To identify 5′ cleavage products and investigate the fate of the deleted U's, reactions identical to those shown in Figure 3A were prepared, but 5′ end–labeled substrate RNA was substituted for 3′ end–labeled RNA. As expected from the 3′ end labeling studies (Figures 2A and 3), a species with the size of edited RNA accumulates over time (Figure 4). A group of products representing 5′ half RNAs can also be seen. The largest of these is derived from cleavage at ES1 and corresponds to the 3′ cleavage product (A in Figure 2A, lane 4). Products one, two, three, and four nucleotides smaller than this species are also observed. These most likely represent molecules that have between one and four U residues removed from the 3′ end of the initial 5′ cleavage intermediate. Interestingly, the smallest species appears to accumulate to a greater extent than the larger species, suggesting that religation of the two half RNAs may be the rate limiting step in the editing reaction. The accumulation of these 5′ cleavage products over time may suggest that some do not go on to form edited product. The total amount of 5′ cleavage products may therefore be comprised of both those molecules that are steady-state intermediates in the productive editing pathway as well as those that are lost

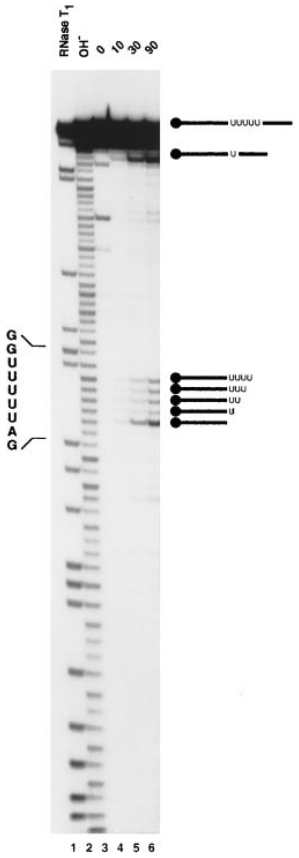

Figure 4. Direct Visualization of Processing Using 5′-End-Labeled Substrate RNA

Substrate RNA was labeled with [α-^{32}P]GTP using guanylyltransferase and subsequently treated with periodate to inhibit the addition of U's to the 3′ end by TUTase activity present in the extract. Aliquots were subjected to partial RNase T1 digestion (lane 1) or to partial alkali hydrolysis (lane 2) to serve as markers, or used in in vitro processing reactions that included gA6[14]Δ16G and were incubated for 0, 10, 30, or 90 min (lanes 3–6, respectively). Input RNA, edited product, and presumptive reaction intermediates are indicated. The sequence of ES1 is shown on the left.

from this pathway and that accumulate over time (see Discussion). When compared with the mobility of partial RNase T1 and alkaline hydrolysis ladders of the input substrate RNA, these products migrate one nucleotide larger than predicted based on characterization of the 5′ terminus of the 3′ cleavage product (see Figure 2C). Therefore, 5′ cleavage products may carry a 3′ hydroxyl group, as has been demonstrated for 5′ cleavage products of other mitochondrial pre-mRNAs (Piller et al., 1995a).

Mutant gRNAs

Chimeric gRNA-pre-mRNA molecules such as those observed in Figures 2A, 3A, and 3B have been proposed to represent RNA editing intermediates (Blum et al., 1991; Cech, 1991; Harris and Hajduk, 1992; and see Introduction, this study). To investigate the significance of the chimeras produced in vitro, several mutant gRNAs were constructed in the background of gA6[14]Δ16G (Figure 5A) and used in processing reactions with 3′ end–labeled substrate RNA (Figure 5B).

Since models involving chimeric intermediates suggest that the oligo(U) tail of the gRNA serves as a repository for the deleted U's, we first investigated the importance of the oligo(U) tail of the gRNA. Removal of the U tail (Figure 5A, No Tail) did not diminish gRNA-directed substrate RNA cleavage and only slightly reduced formation of D-type chimeras. (Compare No Tail [lane 6] with the complete gRNA [lane 4] in Figure 5B.) This mutation more severely diminishes the amount of edited product, as does a mutation that removes even more gRNA sequence (Figure 5B, Trunc. 1, lane 7; note that chimeras formed by Trunc. 1 are shorter, owing to the smaller size of this gRNA), suggesting a role for the oligo(U) tail in edited product formation but not substrate RNA cleavage or chimera formation. The oligo(U) tail has been proposed to recognize purine-rich sequence upstream of processing sites as an alternative role in editing (Blum and Simpson, 1990). To determine if interaction between the gRNA and pre-mRNA 5′ of the processing site was an important determinant for edited product formation, we constructed a mutant gRNA (Figure 5A, Trunc. 4) that strengthens the interaction between the pre-mRNA and gRNA immediately upstream (with respect to the pre-mRNA) of the processing site. This gRNA allows substrate RNA cleavage at wild-type levels and, although formation of edited product is decreased, formation of both types of chimeras is essentially abolished (Figure 5C, lane 3). (Note that the region of the gel expected to contain D-type chimeras predominantly contains pre-mRNA cleavage products, as demonstrated by RNA sequencing [data not shown]). Together, these data suggest that hybridization 5′ of the processing site (with respect to the pre-mRNA) is important for the formation of edited product, but it is not required for (Figure 5B, lanes 6 and 7) and suppresses (Figure 5C, lane 3) chimera formation.

To investigate if the identity of the 3′ terminal nucleotide of the gRNA is an important determinant of processing, a gRNA was constructed that contains the sequence 5′-CUAG-3′ at its 3′ end of an otherwise unaltered molecule (Figure 5A, Xba). This gRNA supports substrate RNA cleavage and the formation of edited product at essentially normal levels (Figure 5B, lane 5). Chimeras are formed at approximately normal levels as well, albeit with a reduced ratio of D-type to E-type chimeras when compared with the reaction containing unaltered gRNA (see Discussion). Thus, the identity of the terminal nucleotide does not seem to be critical for processing.

We next tested the importance of the anchor duplex, since cleavage of the pre-mRNA is directed to the 3′ end (with respect to the gRNA) of this helix. Removal of the gRNA sequence that forms this duplex (Figure 5A, No Anchor) reduces substrate RNA cleavage and chimera and edited product formation (Figure 5B, lane 3) to levels observed without any gRNA (Figure 5B, lane 2), consistent with earlier findings (Blum and Simpson, 1992). A gRNA consisting only of the sequence that forms the anchor duplex (Figure 5A, Trunc. 2) directs nuclease attack between U2 and U1 of ES1 as specifically as does gA6[14]Δ16G, but does not promote the

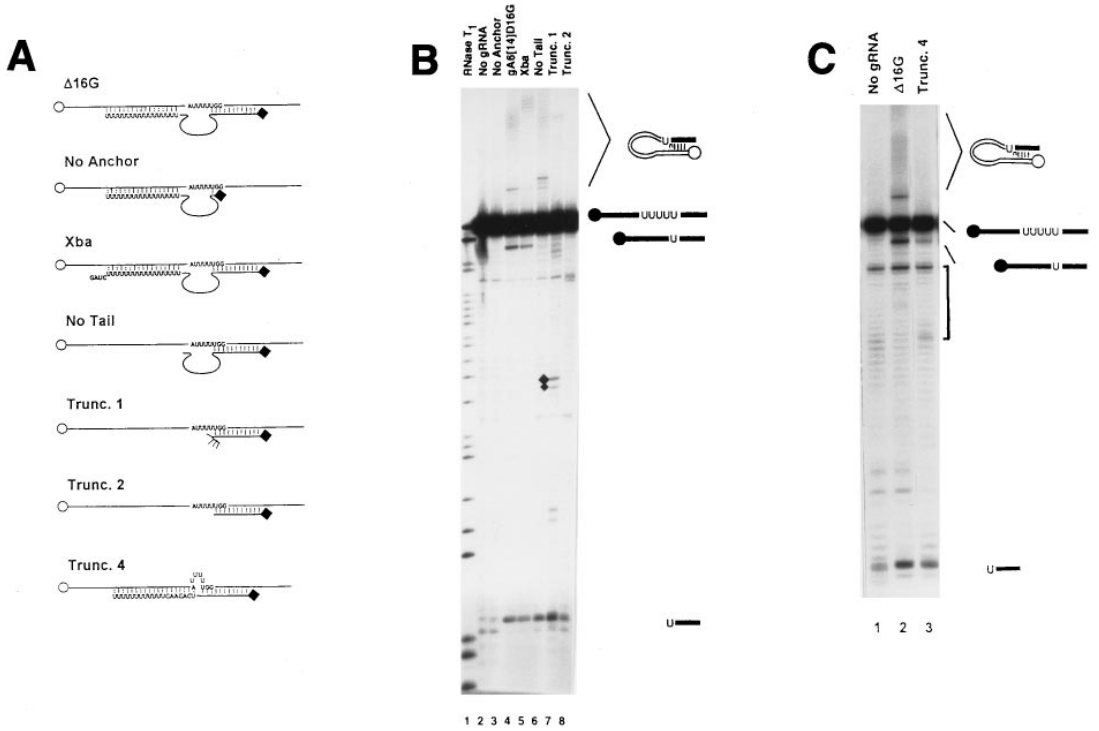

Figure 5. Mutations Define Important gRNA Sequence Elements

(A) Schematic diagram of the gRNA mutations. Editing substrate RNA molecules are shown on the top with open circles representing their 5′ ends. Filled diamonds indicate the 5′ ends of gRNAs. Watson–Crick base pairs are indicated by lines, while G:U base pairs are denoted by colons.

(B) 3′ end–labeled substrate RNA was incubated as in Figure 3A, lane 8 either without gRNA (lane 2), or with No Anchor (lane 3), gA6[14]Δ16G (lane 4), Xba (lane 5), No Tail (lane 6), Trunc. 1 (lane 7), or Trunc. 2 (lane 8). Substrate RNA was subjected to partial RNase T1 digestion and used as a marker (lane 1). Diamonds indicate chimeras formed with the Trunc. 1 gRNA. Note that the E-type chimeras produced with the No Tail gRNA (lane 6) most likely represent chimeras linked at sites upstream of ES1 as demonstrated for some of the chimeras produced with gA6[14]Δ16G in this region of the gel (data not shown). The D-type chimeras formed with the No Tail gRNA (lane 6) are slightly larger than those formed with gA6[14]Δ16G, presumably because additional nucleotides had been added by T7 RNA polymerase during transcription. The identity of the various RNA species produced in vitro is indicated.

(C) 3′ end–labeled substrate RNA was incubated as in Figure 3A, lane 8, either without gRNA (lane 1), or with gA6[14]Δ16G (lane 2), or Trunc. 4 (lane 3). The bracket indicates the region where Trunc. 4/pre-mRNA chimeras linked with various numbers of U's at ES1 are expected to migrate.

formation of chimeras and edited product (Figure 5B, lane 8). Thus, hybridization between the gRNA and pre-mRNA sequence downstream of the processing site (with respect to the pre-mRNA) is necessary and sufficient for efficient initial substrate RNA cleavage.

A Role for the 3′ End of the gRNA

To directly address the importance of the 3′ end of the gRNA in the reaction, we blocked its function and assayed for an effect on processing. Oxidation of the terminal 2' and 3′ hydroxyls of the gRNA with periodate (Figure 6A, lane 4) or replacement of the 3′ OH with a phosphate group by ligation of pUp (lane 5) does not diminish the amount of substrate RNA cleavage relative to untreated gRNA (lane 2) or to mock periodate–treated gRNA (lane 3). Thus, the nucleophilic character of the 3′ OH of the gRNA is not required for pre-mRNA cleavage. However, both means of modification inhibit the formation of D- and E-type chimeras and the formation of edited product. To exclude the possibility that the modifying reagents contaminated the treated gRNAs and caused the observed inhibition, we tested the ability of unmodified gRNA to rescue processing of a reaction containing pUp-blocked gRNA. Rescue would be expected if modification of the gRNA prevented processing but would not be expected if free pUp is responsible for the inhibition. Figure 6B shows that a 1:1, a 2:1, or a 10:1 ratio of untreated gRNA:phosphate–blocked gRNA allows processing (lanes 3, 4, and 5, respectively) at the level observed with exclusively untreated gRNA (lane 6), while the modified gRNA alone (lane 2) inhibits processing.

Discussion

The work described here tested models for the mechanism of RNA editing. We determined whether potential intermediates appeared prior to edited product during time course experiments. We also ascertained whether intermediates had the sequence characteristics predicted by the respective models. Furthermore, to validate the model suggested by our data, we determined whether it was consistent with published data concerning the characteristics of the editing process as surmised both from in vivo and in vitro studies.

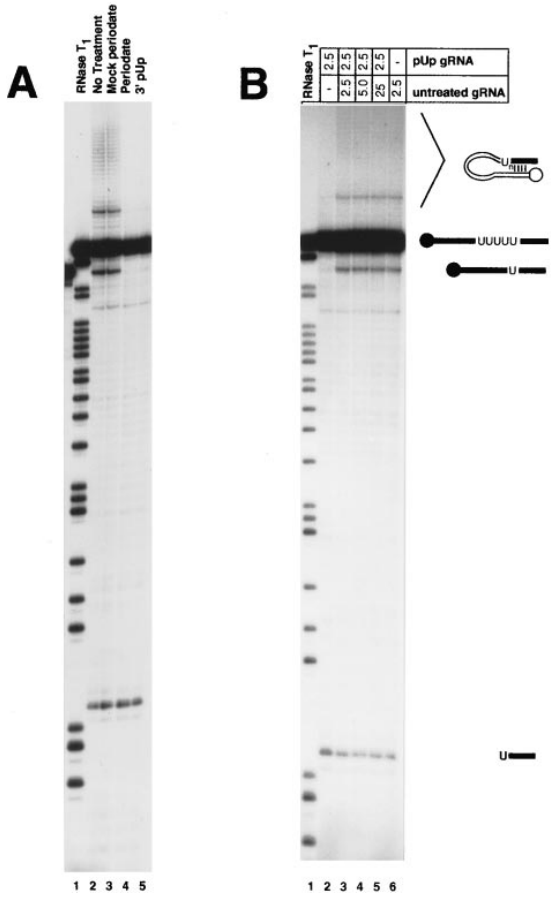

Figure 6. Testing the Importance of the 3′ OH of the gRNA

(A) Reactions identical to those in Figure 3A, lane 8, were carried out except that they contained either untreated gRNA (lane 2), mock periodate–treated gRNA (lane 3), periodate-treated gRNA (lane 4), or gRNA with pUp ligated to its 3′ end (lane 5). Substrate RNA was subjected to partial RNase T1 digestion and used as a marker (lane 1).

(B) Reactions were carried out as above but contained either 2.5 pmol of pUp-modified gRNA (lanes 2–5) and 2.5 pmol (lanes 3 and 6), 5 pmol (lane 4), or 25 pmol (lane 5) of unmodified gRNA. Substrate RNA was subjected to partial RNase T1 digestion and used as a marker (lane 1).

Are Chimeras Intermediates?

The appearance of potential intermediates relative to edited product in time course experiments provides data critical for testing mechanistic models of U deletion. The 3′ half RNA (labeled A in Figure 2A) could be an intermediate in the formation of edited RNA, since the former appears prior to the latter (Figure 3B). In addition, the attainment of steady-state levels by the 3′ cleavage product, as the level of edited product increases, is consistent with an intermediate. The 5′ half RNAs also may be intermediates, since the largest of these is probably produced by the same cleavage event that generated the 3′ half RNA and the smaller ones are probably derived from it by removal of U's from its 3′ end (Figure 4). In contrast, chimeras both with and without multiple U residues linking the gRNA and the substrate RNA (E and D classes, respectively) appear coincident with, or subsequent to, edited RNA (Figure 3). The accumulation of edited RNA and chimeras is consistent with both being in vitro end products. Therefore, the chimeras we detect in vitro do not seem to be intermediates by this criterion.

The sequence characteristics of the cleavage products and chimeras also suggest that only the former are intermediates. An element of models suggesting chimeras are intermediates is that gRNAs serve as a repository for U's deleted by editing (Blum et al., 1991; Cech, 1991; Harris and Hajduk, 1992). The sequences of D-type chimeras indicate that they do not contain enough U's to account for those deleted from the substrate RNA, and so they cannot serve as a repository for U's. The chimeras containing multiple U's linking gRNA and pre-mRNA (E-type) also do not seem to function as repositories for the deleted U's, since these residues appear to be sequentially removed from the 3′ end of the 5′ cleavage product (Figure 4). The cleavage–ligation model proposing chimeric intermediates predicts that the 3′ cleavage product should carry these U's, while the transesterification model predicts that neither cleavage product should carry them (Blum et al., 1991; Cech, 1991). In fact, the existence of 3′ cleavage intermediates is inconsistent with the transesterification model (Blum et al., 1991; Cech, 1991). However, since the 3′ end of the gRNA is required for edited product formation (Figure 6), we cannot exclude the possibility that chimeras that escape detection or that do not serve as a repository for U's are intermediates. Nevertheless, the characteristics of the observed D-type chimeras and the observed substrate RNA cleavage products argue against both models that suggest that chimeric molecules serve as a repository for U's during the deletion editing reaction, thereby eliminating an attractive feature of these models.

The Role of the gRNA Oligo(U) Tail

Chimeras are produced in our in vitro system, as well as in others (Harris and Hajduk, 1992; Koslowsky et al., 1992; Blum and Simpson, 1992), and are present in kinetoplastid mitochondrial RNA (Blum et al., 1991; Read et al., 1992; Arts et al., 1993). How can their existence be explained, since they do not seem to be intermediates in vitro? As an alternative to functioning as a repository for U's, the oligo(U) tail of the gRNA has been proposed to associate with purine-rich sequences upstream of editing sites (Blum and Simpson, 1990). We propose that this interaction is (partly) responsible for holding the 3′ region of the 5′ cleavage product near the catalytic center of the editing machinery, much as conserved U residues of the U5 snRNA hold the 5′ cleavage product during pre-mRNA splicing (reviewed by Sharp, 1994). Destabilization of this interaction could allow the 3′ end of the gRNA to occupy the position of the 5′ cleavage product and be utilized in a non-productive editing pathway (see below). The continuous accumulation of 5′ cleavage products during the in vitro reaction (Figure 4), but not of the 3′ cleavage product (Figure 3), supports the notion that a fraction of the 5′ cleavage products is lost from the catalytic center of the complex. A differential requirement for the oligo(U) tail/pre-mRNA interaction in the production of edited RNA and chimeras as demonstrated by the mutant gRNAs (Figure 5), is consistent with this proposal. This interpretation of the role for

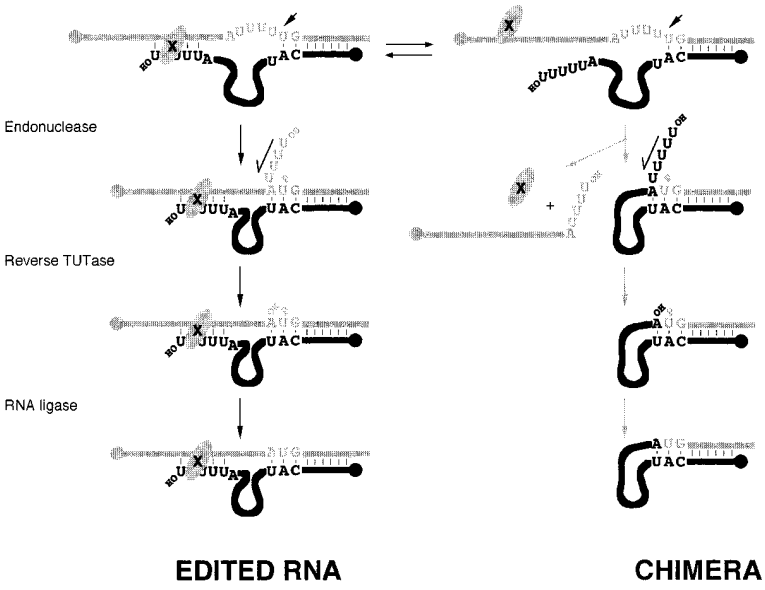

Figure 7. A Model for U Deletion

gRNA is shown in black and pre-mRNA in gray. Important nucleotide sequence elements of gRNA and pre-mRNA are indicated. The productive deletion pathway is shown on the left panel. Catalysis begins with gRNA-directed substrate RNA cleavage at the 3′ end (with respect to the gRNA) of the anchor duplex. Pairing interactions may then force the U's on the 5′ cleavage product into a configuration where they are susceptible to exonucleolytic removal. The 5′ half RNA may be held in place by the oligo(U) tail of the gRNA and an unidentified factor (X) while the 3′ cleavage intermediate is held by the anchor duplex. Basepairing of the gRNA with the two halves of the pre-mRNA aligns them for ligation. If the interaction between the oligo(U) tail of the gRNA and the 5′ cleavage intermediate is disrupted, the latter may be lost and the 3′ end of the gRNA may become a substrate for U removal and ligation resulting in chimera formation (right diagram).

the oligo(U) tail is compatible with the sequences of all chimeras observed including those that have variable numbers of U's linking gRNA with pre-mRNA and those that are truncated in the gRNA portion (Blum et al., 1991; Blum and Simpson, 1992; Harris and Hajduk, 1992; Koslowsky et al., 1992; Read et al., 1992; Arts et al., 1993).

The Significance of the 3′ End of the gRNA

The appropriate chemical nature of the 3′ end of gRNA is essential in vitro for production of edited RNA (Figure 6) and chimeras (Harris and Hajduk, 1992; Koslowsky et al., 1992; and see Figure 6, this study), since modification of gRNA 3′ ends by periodate oxidation or ligation with pNp blocks both processes. These results suggest that edited RNA and chimeras are formed by the same process and could imply that chimeras are intermediates in the production of edited RNA, as has previously been proposed (Blum et al., 1991; Cech, 1991; Harris and Hajduk, 1992). However, since chimeras do not display all the characteristics that would be expected for intermediates (see above), alternative roles for the 3′ end of gRNAs, besides direct involvement in the catalytic step(s) of editing, are possible. For example, the 3′ end may be an essential part of a recognition domain for binding of a component of the editing machinery, analogous to the 3′ hydroxyl group of the U6 snRNA in directing the binding of the nuclear antigen La in Xenopus oocytes (Terns et al., 1992). Moreover, in T. brucei the specific crosslinking of a 124 kDa mitochondrial protein to gRNA has been suggested to require its 3′ end (Köller et al., 1994). This could provide a possible explanation for the importance of the 3′ end of the gRNA in RNA editing. However, further work is needed to resolve this issue.

A Mechanism for Editing

We interpret our data to suggest the model for RNA editing shown in Figure 7. By this model, editing is initiated when the gRNA directs nuclease attack at the 3′ end (with respect to the gRNA) of the anchor duplex (Figure 2C). Lanes 3 and 8 of Figure 5B show that the anchor duplex alone is both necessary and sufficient for this first step. Cleavage leaves a 5′ monophosphate on the 3′ cleavage product (Figure 2C) and a 3′ hydroxyl on the 5′ cleavage product (Figure 4) (Piller et al, 1995a). The U's to be deleted are carried on the 3′ end of the 5′ cleavage product (Figure 4), which may be held in place by the oligo(U) tail of the gRNA (Figure 5B). In the productive editing pathway, coaxial stacking forces could then extend the anchor duplex by pairing the first adenosine upstream of the processing site in the pre-mRNA to the U in the guiding sequence of the gRNA. If the 5′ cleavage product does not occupy its appropriate position, the A at the base of the oligo(U) tail of the gRNA may take its place (Figure 7). In both cases, this positioning would extrude the U's to be deleted from the helix, and facilitate their recognition and removal. The U residues appear to be removed sequentially from the RNA in this position (probably as free 5′-uridine monophosphate), since a set of 5′ half pre-mRNAs differing in the number of U residues at their 3′ ends are generated (Figure 4). During productive editing, coaxial stacking may also serve to align the 3′ cleavage product and the processed 5′ cleavage product during the ligation of the two half RNAs or, in the aberrant pathway, to align the 3′ end of the gRNA and the 5′ end of the 3′ cleavage product to form a D-type chimera. Except for its accommodation of chimeras, this model is very similar to the initial proposal for the mechanism of RNA editing (Blum et al., 1990) and can accommodate the proposals for editing site selection within a gRNA-specified block of sequence (Koslowsky et al., 1991; Sturm et al., 1992).

The enzymatic activities required in our model exist in the mitochondria of kinetoplastids. Endonuclease, TUTase, and RNA ligase activities in T. brucei (Pollard et al., 1992) and an activity that catalyzes internal U incorporation in Leishmania tarentolae (Peris et al., 1994) reside in an ~20S complex. Since the U deletion machinery also occurs in an ~20S particle (R. A. Corell et al.,

1996), these enzymatic activities are probably present in the complex active in U deletion and, as predicted by our model, probably involved in the reaction. Previously, it has been shown that endonuclease present in this complex cleaves several pre-mRNAs near the 3′ end of editing domains in a gRNA-independent fashion (Harris et al., 1992; Simpson et al., 1992; Piller et al., 1995b), and we see a low level of gRNA-independent cleavage in the vicinity of ES1 (Figures 2A and 5C). Sites subject to gRNA-independent cleavage are thought to be positioned in a loop in an intramolecular stem-loop structure formed by the pre-mRNA (Piller et al., 1995a). We propose that pre-mRNA cleavage in the productive editing pathway is gRNA directed, and that the intramolecular pre-mRNA stem-loop may simply mimic the gRNA/pre-mRNA anchor duplex in vitro to allow endonuclease cleavage. U removal from the 3′ end of the 5′ cleavage product (Figure 4) or the gRNA could be accomplished by TUTase activity present in the ~20S particle that operates in reverse. Alternatively, a U-specific 3′ exonuclease may be responsible for U removal, as has been suggested previously (Blum and Simpson, 1990; Blum et al., 1990). The formation of a 3′ cleavage product with a 5′ monophosphate is consistent with the involvement of an RNA ligase in the reaction, since it has been demonstrated that the RNA ligase found in 20S fractions utilizes this moiety (Rusché et al., 1995; Sabatini and Hajduk, 1995). In addition, the requirement for hydrolysis of the α–β phosphate bond of ATP during deletion editing (Seiwert and Stuart, 1994), also a requirement for RNA ligase, is consistent with an involvement of RNA ligase in editing (Rusché et al., 1995; Sabatini and Hajduk, 1995, and references therein).

Strong support for the notion that chimeras are formed in an aberrant pathway in which the oligo(U)/pre-mRNA interaction becomes destabilized is supplied by gRNA mutations. A mutation that strengthens the interaction between the gRNA and the pre-mRNA upstream of the processing site (Figure 5A, Trunc. 4) allows formation of edited product but dramatically reduces chimera formation (Figure 5C, lane 3), while those that weaken it have the opposite effect (Figure 5B, No Tail and Trunc. 1). Several observations further suggest that the 3′ end of the gRNA mimics the 3′ end of the 5′ cleavage product. First, the number of U's linking the gRNA and substrate RNA in the D-type chimeras is programmed by the gRNA sequence at the processing site. Second, a gRNA with non-U 3′ terminal nucleotides (Figure 5A, Xba) results in a decreased relative ratio of D-type to E-type chimeras (Figure 5B, lane 5) as would be expected if the terminal nucleotides are removed by a 3′ exonuclease that has a preference for U's. Finally, gRNAs that are chemically blocked at their 3′ ends are unable to form D-type chimeras (Figure 6A). Despite this analogy, chimera formation cannot require precise basepairing at the processing site since E-type chimeras are derived from gRNAs with a 3′ terminal U. Their formation could occur within the complex or simply be the result of RNA ligase present in the extract acting on free gRNA and 3′ cleavage product (Rusché et al., 1995; Sabatini and Hajduk, 1995).

In vivo, edited RNA is much more abundant than chimeric molecules (Riley et al., 1995). The relatively high abundance of chimeras in our in vitro system may suggest that in vitro conditions do not precisely reproduce those in vivo. The ability of higher levels of divalent cations (which could stabilize the oligo(U) tail/substrate RNA interaction) or the addition of carrier RNA (which could competitively inhibit excess reverse TUTase activity) to increase the relative ratio of edited product to chimeras indicates that this ratio is plastic and not an inherent property of the reaction(s) (data not shown). In addition, a component that influences this ratio (labeled X in Figure 7) may be lost from the ~20S complex during extract preparation.

By extrapolation, our model suggests a mechanism for U addition, the more frequent form of kinetoplastid RNA editing. During this reaction gRNA would direct cleavage of the pre-mRNA as it does during the deletion reaction. Purines in the gRNA across from the processing site would then direct TUTase-mediated U addition to the 3′ OH of the 5′ cleavage product. As with deletion, the gRNA would align and juxtapose for ligation the 3′ cleavage product and the 5′ cleavage product when the appropriate number of U's is attained. We have recently demonstrated gRNA specified addition of U's to pre-mRNA in vitro and indeed observe the intermediates predicted by our model (M. Kable et al., unpublished data). Furthermore, as predicted by our model, free UTP is required for addition editing, and UMP is added to the 3′ end of the 5′ cleavage product (M. Kable et al., unpublished data).

Evolutionary Implications

RNA editing has been proposed to proceed by a self-catalyzed mechanism fundamentally similar to RNA splicing reactions (Blum et al., 1991; Cech, 1991). The work described herein suggests that the putative intermediates in the self-catalyzed pathway are nonproductive end products of the reaction, and other work suggests that protein(s) present in the ~20S glycerol gradient fractions carry out the reaction, not the RNAs themselves (S. D. S., unpublished data). Thus, RNA editing does not appear to be mechanistically related to intron removal. The suggested similarity between these two processes raised the possibility that both were derived from a very ancient process present in the "RNA world." Our work argues against this possibility, but does not bear directly on the time of origin of RNA editing.

Experimental Procedures

Production of RNAs

A6short/TAG.1 was constructed by polymerase chain reaction (PCR) using A6/TAG.1 (Seiwert and Stuart, 1994) as a template and oligonucleotides A6short (5′-GTAATACGACTCACTATAGGAAAGGTTA GGG-3′) and A6-TAG.1 (5′-GCGCGTCTAGATGCCAGGTAAGTATTC TATAACTCCAAAAATC-3′). gA6[14]Δ16G and gA6[14] were produced as described (Seiwert and Stuart, 1994). The template for "Xba" gRNA was produced by mutagenic PCR of a gA6[14] clone containing an Xba site at its 3′ end (Göringer et al., 1994) using oligonucleotides T7A6–3 (5′-GTAATACGACTCACTATAGGATATAC TATAACTCCATAACGAATC-3′) and T3 (5′-ATTAACCCTCACTAAA GGG-3′) (which anneals 3′ of polylinker sequence on the downstream side of the clone). The template for "No Anchor" gRNA was produced by mutagenic PCR of a PCR product encoding

gA6[14]Δ16G (Seiwert and Stuart, 1994) using oligonucleotides -Anchor (5′-GTAATACGACTCACTATAGATAACGAATCAGATTTTG AC-3′) and T3. A template for the gRNA "No Tail" was produced by mutagenic PCR of gA6[14]-3′del (Read et al., 1994) with oligonucleotides T7A6–3 and T3. A template for the gRNA "Trunc. 4" was produced by mutagenic PCR of the PCR product encoding gA6[14]Δ16G with oligonucleotides T7 and trunc4 (5′-AAAAAAAAAA AAGTTGTGATGGAGTTATAGTATATC-3′). Trunc. 1 and Trunc. 2 were made by Milligan (Milligan et al., 1987) transcription of oligonucleotides gA6–3Trunc1 (5′-GTTATGGAGTTATAGTATATCCTATAGT GAGTCGTATT AC-3′) or gA6–3Trunc2 (5′-TGGAGTTATAGTATATCC TATAGTGAGTCGTATTAC-3′) and T7. Before runoff transcription with T7 RNA polymerase, Xba, No Anchor, and No Tail PCR products were digested with XbaI, DraI and FokI, respectively. All synthetic RNAs were transcribed with T7 RNA polymerase and gel-purified on 10% polyacrylamide, 7 M urea, 1× Tris–borate–EDTA gels prior to use.

Labeling and Modification of In Vitro Transcribed RNAs

Substrate RNA was 3′ end–labeled [40 pmol RNA, 90 μCi (3000 Ci/mmol) [5′-^{32}P]pCp and 45 U T4 RNA ligase (BRL) in 20 μl reactions containing 25 mM HEPES (pH 8.3), 2.5 mM $MgCl_2$, 10% dimethyl sulfoxide, 15% glycerol, 1.6 mM dithiothreitol, and 25 μM ATP] or 5′ end–labeled [40 pmol RNA, 100 μCi (3000 Ci/mmol) [α-^{32}P]GTP and 25 U guanylyltransferase (BRL) in 50 μl reactions containing 50 mM Tris–HCl (pH 7.9), 1.2 mM $MgCl_2$, 6 mM KCl, and 2.5 mM dithiothreitol]. Radiolabeled RNA was purified on 7 M urea, 9% polyacrylamide, 1× Tris–borate–EDTA gels to separate the transcript of the expected size from those with an additional nonencoded nucleotide at their 3′ ends.

Partial RNase digests using B. cereus RNase (Pharmacia), which cuts 3′ of C and U residues, and RNase U2 (Pharmacia), which cuts 3′ of A residues were done according to the manufacturer's recommendations. Partial digests with RNase T1 (Boehringer Mannheim), which hydrolyzes RNA 3′ of G residues, were done for 15 min at 55°C in 5 μl reactions containing 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 4.2 M urea, 0.6 μg/μl Torula yeast RNA, 0.02% bromophenol blue, 0.02% xylene cyanole, and 0.3 units enzyme. For partial alkaline hydrolysis (hydroxyl ladder), 0.25 pmol end-labeled RNA was incubated for 10 min at 90°C in 50 mM sodium carbonate buffer (pH 9). Phosphatase treatment of RNA was performed using shrimp alkaline phosphatase (USB) according to the manufacturer's specifications.

To oxidize the terminal 2′ and 3′ hydroxyls of gA6[14]Δ16G or 5′-labeled substrate RNA, 100 pmol of RNA was treated with 10 nmol of sodium-m-periodate in 100 mM NaOAc (pH 4.5) for 2 hr at 4°C in the dark. Sucrose (7 mmol) was then added to quench the reaction, and the modified RNA was precipitated with ethanol and gel purified. To phosphorylate the 3′ hydroxyl of gA6[14]Δ16G, pUp was first made by phosphorylation of Up with ATP using T4 polynucleotide kinase. After heat inactivation, this mixture was substituted for [5′-^{32}P]pCp in reactions identical to the 3′ labeling reactions described above, except that 100 pmol of the gRNA replaced for 40 pmol of A6short/TAG.1 RNA. The RNA was gel purified and subjected to another round of ligation to pUp. Both periodate treatment and pUp ligation were >98% effective in blocking as assayed by subsequent [5′-^{32}P]pCp labeling of the gRNA.

In Vitro Reactions and Glycerol Gradients

Glycerol gradients were poured and run as described (Pollard et al., 1992), except that 2–4 × 10^{10} cell equivalents were loaded onto each gradient. From the top of the gradients, 0.5 ml fractions were collected and frozen. In in vitro reactions, 5–20 μl of the samples were used directly in order to determine which fractions contain the highest activity. Typical reactions included 0.25 pmol radiolabeled-substrate RNA, 2.5 pmol gRNA, ~1–5 μg of protein from pooled ~20S fractions containing the highest activity, 0.5 μg Torula yeast RNA, and 5 mM $CaCl_2$ (both of which increase the relative ratio of edited product to chimeras) and were carried out as described (Seiwert and Stuart, 1994) for 40 min to 1 hr unless otherwise indicated. RNAs were collected by extraction with phenol and precipitation with ethanol. Samples were run on 9% polyacrylamide, 7 M urea, 1× Tris–borate–EDTA gels, which were dried and exposed for autoradiography.

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