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The specificity of nucleotide removal during RNA editing in *Trypanosoma brucei*

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ABSTRACT

RNA editing in *Trypanosoma brucei* produces mature mRNAs by posttranscriptional insertion and deletion of uridyates (Us) by a series of catalytic steps, which include endoribonucleolytic cleavage, 3' terminal addition or removal of Us, and RNA ligation. Preedited mRNA (pre-mRNA) and guide RNA (gRNA) that are mutated at or near the editing site (ES) were used to examine the effects on the specificity of in vitro editing. Sequences that are not predicted to form a gRNA/pre-mRNA base pair immediately 5' to the ES still supported accurate editing. Substitution of a non-U nucleotide at various positions within a stretch of Us that are normally removed from the ES resulted in deletion of only the Us that were 3' to the substituted nucleotide. Overall, ES selection by the endoribonuclease, the specificity of the 3' exonuclease for Us, and ligation appear to act in concert to ensure the production of accurately edited RNA.

Keywords: 3' exonuclease; gRNA; mechanism; uridyate specificity

INTRODUCTION

RNA editing is a type of posttranscriptional RNA processing that occurs in the mitochondrion of trypanosomatids (Benne et al., 1986). It inserts and, at a lower frequency, deletes uridyates (Us) to produce mature mitochondrial mRNAs (Stuart et al., 1997). The production of the mature, fully edited mRNAs is developmentally regulated, as is the production of the mitochondrial respiratory system (Feagin et al., 1987; Feagin & Stuart, 1988; Corell et al., 1996). Thus, editing appears to participate in controlling the switch between two alternate energy-generating systems during the life cycle.

A class of small RNAs, called guide RNAs (gRNAs), specifies the edited sequence (Blum & Simpson, 1990; Blum et al., 1990). The gRNAs contain 10–12 nt of “anchor sequence” near their 5' end that are complementary to the pre-mRNA sequence immediately 3' to the sites that become edited. The central informational or guiding region of the gRNA appears to specify the edited sequence, as it is complementary to the fully edited, but not preedited, mRNA. The 3' end of the gRNA contains an oligouridyate tail that is added post-

transcriptionally, possibly by the 3'-terminal uridyate transferase (TUTase) activity that has been detected in kinetoplastid mitochondria (Bakalara et al., 1989; Pollard et al., 1992). The gRNAs are primarily encoded in the minicircle components of the mitochondrial DNA of kinetoplastids (termed kinetoplast DNA or kDNA) whereas the pre-mRNAs are encoded in the maxicircle component of the kDNA. The sequences of some extensively edited mRNAs such as cytochrome oxidase III (Feagin et al., 1988) are more the result of editing than transcription, as more than half of the nucleotides in the mature mRNA were added posttranscriptionally. Despite the extensive sequence remodeling by editing, precise mRNA sequences are produced.

The general process of editing occurs by a series of separate catalytic steps (Seiwert & Stuart, 1994; Kable et al., 1996; Seiwert et al., 1996). The first step is the endoribonucleolytic cleavage of the pre-mRNA, which selects the site that will be edited. This appears to occur at a single-stranded region of the pre-mRNA that is adjacent to a duplex that is formed with the cognate gRNA (Kable et al., 1996; Seiwert et al., 1996). The second step is the addition or removal of Us from the 3' end of the 5' cleavage product of the pre-mRNA, depending on whether it is an insertion or deletion site. The final step of editing of a site is the ligation of the two portions of the pre-mRNA. This may be catalyzed

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by the RNA ligase activity that has been detected in mitochondria of kinetoplasts (Sabatini & Hajduk, 1995; Corell et al., 1996; Piller et al., 1996) and that is encoded in recently identified genes (McManus et al., 2001; Panigrahi et al., 2001; Rusché et al., 2001; Schnauffer et al., 2001). Although all these appear to be steps in RNA editing, they do not reveal how the edited sequence is actually specified. Specifically, how does the gRNA specify the number of Us that are actually inserted or deleted? The question of how precision is achieved is crucial because the removal or insertion of even one additional nucleotide would lead to a frame-shift and thus to the production of nonfunctional mRNA.

We used the *in vitro* editing system that was developed in this laboratory (Seiwert & Stuart, 1994; Seiwert et al., 1996) to explore the process of how the number of Us that are deleted is specified during RNA editing. We found that the predicted base pair between the gRNA and the pre-mRNA nucleotide immediately 5' to the editing site (ES) is not required for deletion editing, as did others (Cruz-Reyes et al., 2001). This eliminates a hypothesis that such a base pair is needed to align the appropriately processed 5' cleavage product of pre-mRNA for ligation with the 3' cleavage product. We also found that only Us are deleted from an ES during *in vitro* editing. This indicates that the 3' exoribonuclease that functions in RNA editing is U-specific (i.e., is a 3' exouridylylase). This specificity along with selection of the ES by the endoribonuclease and perhaps preferential ligation at the ligation step appear to determine the number of Us that are removed at a deletion ES.

RESULTS

Base pairing immediately 5' to the ES is not required for accurate deletion editing

Understanding the specificity of each step in the editing reaction series (i.e., endonucleolytic cleavage, U addition/removal, and ligation) is crucial to elucidating the mechanism by which editing results in the precise RNA sequence. The ligation step may contribute to accurate editing by preferential ligation to the 3' cleav-

age products of 5' cleavage products whose 3' termini have been correctly processed, as suggested by Kable et al. (1996) and Igo et al. (2000). Based on this suggestion and the recognition that base-pair formation between the gRNA and pre-mRNA immediately 5' to the ES occurs in all cases of *in vivo* editing known led Kable et al. (1996) to suggest a model in which base pairing between mRNA and gRNA both 5' and 3' to the ES is critical for accurate editing. To test the hypothesis, we prepared pre-mRNA substrates and/or cognate gRNAs that are mutated immediately 5' to the ES and tested their ability to generate accurately edited RNA *in vitro*.

The gA6[14]Δ16G gRNA specifies the deletion of four Us from the first (i.e., most 3') editing site (ES1) of pre-edited A6U5 substrate (Seiwert & Stuart, 1994; Seiwert et al., 1996; Fig. 1). Incubation of these RNAs with mitochondrial extract resulted in the production of cleaved substrate (only the 3' fragment is visualized with 3' end-labeled substrate), edited RNA from which four Us are deleted, and chimeric molecules that contain the 3' cleavage fragment of the pre-mRNA appended to gRNA (Fig. 2A, lane 3; Seiwert et al., 1996). Edited RNAs, chimeras, and cleaved substrates were not produced when the gRNA was omitted, showing that their production is dependent on the gRNA (Fig. 2A, lane 2).

Several combinations of gRNA/substrate pre-mRNA pairs with various (single-nucleotide) mutations were used to examine the requirement in editing for a base pair immediately 5' to the ES. Specifically, gRNAs with mutations at position U₁₉ and substrate pre-mRNAs with mutations at position A₄₁ were produced and tested in pairwise combinations for their ability to support deletion editing *in vitro*. Some of these gRNA/substrate pairs are predicted to form Watson–Crick or G•U base pairs immediately 5' to the ES whereas others are not. Pairs with upstream nucleotides G₄₁•U₁₉ and G₄₁-C₁₉, which are predicted to form a base pair upstream of the ES as a consequence of compensatory mutations, resulted in the production of the predicted 3' cleavage product, chimeras, and edited RNA (Fig. 2A, lanes 8 and 10). However, some combinations that could not

FIGURE 1. pre-mRNA and gRNA sequences. Shown are A6U5 substrate RNA and gA6[14]ΔG gRNA (Seiwert & Stuart, 1994) annealed at the anchor duplex as indicated. Endonucleolytic cleavage sites for ES1 directed by wt gA6[14] (wt) and gA6[14]ΔG (ΔG) are indicated by arrows. The four Us in reverse color (white on black) are deleted during editing at ES1 directed by gA6[14]ΔG. The potential base pair upstream of ES1 between A₄₁ of A6U5 and U₁₉ of gA6[14]ΔG is indicated by a dashed line.

FIGURE 2. Upstream base pair interaction. **A:** PhosphorImager (Molecular Dynamics) depiction of products of in vitro editing reactions separated on a 9% (19:1) denaturing polyacrylamide gel run in 1× TBE. A6U5 was mutated at position 41 from A to G or C, and gA6[14]ΔG was mutated at position 19 from U to G or C. The pCp 3' end labeled in vitro-transcribed pre-mRNA and gRNA were incubated with a 20S glycerol gradient fraction from *T. brucei* mitochondrial extract in reaction buffer described in Seiwert and Stuart (1994). Nucleotides at position 41 in mRNA and at position 19 in gRNA are indicated above the lanes. Lanes 1, 6, and 11 (T₁) contain labeled A6U5, mG₄₁, and mC₄₁ RNA, respectively, partially digested with RNase T₁ and included as a size marker. Note the extra T₁ digestion product cleaved at G₄₁ in lane 6, lower panel. Lanes 2, 7, and 12 (–) are reactions lacking gRNA. The input mRNA (Input), edited RNA with four Us deleted (Edited), and 3' fragment of endonucleolytic cleavage at ES1 (3'CL (ES1)) are indicated by arrows. A brace indicates chimeras of gRNA and the 3' cleavage fragment. **B:** Diagram showing probable association of mG₄₁ pre-mRNA and gG₁₉ gRNA. The 5' terminus of the anchor duplex is shown, and normal cleavage site at ES1 is indicated by an arrowhead. An arrow shows the site of cleavage resulting in the 3' fragment in **A**, lane 9, lower panel. The dotted line represents a potential base pair between G₄₁ of mG₄₁ and C₂₂ of gG₁₉ that could direct cleavage upstream of G₄₁.

result in base-pair formation at the designated site, such as A₄₁/C₁₉, C₄₁/U₁₉, and C₄₁/C₁₉, also resulted in the production of 3' cleavage product, chimeras, and edited RNA (Fig. 2A, lanes 5, 13, and 15, respectively). This implies that a Watson–Crick or G•U base pair between gRNA and pre-mRNA immediately 5' to the editing site is not required for accurate deletion editing in vitro.

Substitution of G at position 19 in the gRNA predicts extension of the anchor duplex by 3 bp with any of the above pre-mRNAs (Fig. 2B). Incubation of this gRNA with any of these pre-mRNAs did not result in cleavage at ES1 or immediately 5' to the predicted extended duplex (Fig. 2A, lanes 4, 9, and 14). However, cleavage occurred 5 nt 5' to ES1 upon incubation of this gRNA (gG₁₉) with mG₄₁ pre-mRNA, as is apparent from the 3' cleavage fragment and chimeras that are larger than

those resulting from cleavage at ES1 (Fig. 2A, lane 9). A minor cleavage product of the same size was also detected upon incubation of gA6[14]ΔG (gU₁₉) with the same substrate (Fig. 2A, lane 8), but not with the other gRNA/pre-mRNA combinations. This implies that base pairing of the G at position 41 of mG₄₁ with C or U at position 19 in the gRNAs (see Fig. 1) and interactions between pre-mRNA and gRNA upstream of the cleavage site and/or interaction with the editing complex contributed to cleavage at this site.

The kinetics of accumulation of the products of the editing reactions were examined to assess possible preferential effects on some editing steps because lack of a base pair immediately 5' to the ES did not affect the final outcome of editing (Fig. 2A, lanes 5, 13, and 15). A higher rate of cleavage and greater accumulation of chimeras were observed with nucleotide pairs

A₄₁-U₁₉ compared to C₄₁/U₁₉, although a similar amount of edited RNA accumulated with both substrates (data not shown). Thus, a base pair immediately upstream of the editing site is not required for accurate deletion editing in vitro and does not affect the overall rate of editing.

The results of the studies on the importance of the base pair 5' of the ES are summarized in Table 1. The nucleotide combinations A₄₁-gU₁₉, G₄₁•U₁₉, and G₄₁-C₁₉, which can form upstream base pairs, resulted in editing, perhaps not surprisingly. However, the A₄₁/C₁₉, C₄₁/U₁₉, and C₄₁/C₁₉ combinations, which are not predicted to form Watson-Crick or G•U base pairs, also resulted in editing, showing that base pairing of the pre-mRNA/gRNA nucleotides immediately 5' to the ES is not required for accurate deletion editing. Substitution of gG₁₉ (or gA₁₉; data not shown) ablated editing at ES1, apparently as a result of base pairing with some of the Us that are normally deleted, thus preventing cleavage or shifting the site of cleavage further upstream.

The 3' exonuclease is U specific

Because base pairing immediately 5' to ES1 does not appear to be required for precise deletion of uridines from ES1, another step(s) must direct removal of the precise number of Us specified by gRNA. One possibility is that the exoribonuclease that removes nucleotides from an ES may be specific to Us. Although that has been a presumed characteristic of the exonuclease (Cruz-Reyes & Sollner-Webb, 1996), and although a 3' exoUase has been partially purified in *Leishmania tarentolae* mitochondria (Aphasizhev & Simpson, 2001), the U specificity of nucleotide removal has not been systematically tested in an in vitro editing context. Following the endoribonucleolytic cleavage, such an exonuclease would be predicted to remove the Us from the 3' end of the 5' cleavage product until it encounters non-U nucleotides, after which the 5' and 3' cleavage products would be ligated to complete one round of editing. To test this possibility, we constructed a series of pre-mRNA substrates in which the Us in the ES were

replaced one by one with a non-U nucleotide (Fig. 3A) and analyzed their processing in vitro. Two gRNAs, gA6[14]ΔG and wt gA6[14] gRNA, were used to verify the gRNA-directed processing of the mutated pre-mRNAs. Processing was compared to previously characterized editing of A6U5 pre-mRNA, which is edited by the removal of four or three Us using the gA6[14]ΔG or wt gRNAs, respectively (Fig. 3B,C, lanes 11 and 12; also see Seiwert & Stuart, 1994). The pre-mRNA 3' cleavage product directed by wt gRNA is 1 nt longer than with gA6[14]ΔG, because the anchor duplex is 1 bp longer, which shifts the cleavage site 1 nt upstream.

In vitro incubation of mC₄₂ substrate and wt gRNA or gA6[14]ΔG resulted in edited RNAs that are 2 and 3 nt shorter than the input substrate, respectively (Fig. 3B, lanes 9 and 10). This is the size expected if the C introduced at position 42 is not deleted. The sequence of the edited RNA in Figure 3B, lane 10, was confirmed by performing a preparative scale reaction and performing enzymatic sequencing on the isolated products (data not shown). The cleavage sites and chimera populations seen with these gRNAs remained unchanged compared to when incubated with the control (A6U5) RNA (Fig. 3B, compare lanes 9 to 11 and 10 to 12). Incubation of mC₄₃ with the same gRNAs resulted in edited RNAs in which 1 and 2 nt, respectively, are deleted from the editing site. This is the size expected if the introduced C and the U upstream of this C are not deleted. Again, the cleavage sites and chimera populations remained unchanged (Fig. 3B, lanes 7 and 8). Similarly, incubation of mC₄₄ pre-mRNA also appears to produce edited RNA from which the U that is 3' to the introduced C was deleted when incubated with gU₁₉. The presence of the 3' cleavage and chimera products suggests that the edited product was produced. However, this product would be obscured by a band of identical size that is present in all lanes, including that of the negative control, likely due to incomplete purification of the two T7-transcribed RNAs. No edited RNA, 3' cleavage product, or chimeras were produced with mC₄₄ substrate and wt gRNA combination, presumably because the anchor duplex is extended through the editing site (Fig. 3B, lane 5). Also, incubation of mC₄₅ substrate with either gRNA did not result in apparent edited RNA products (Fig. 3B, lanes 3 and 4). However, a smaller gRNA-dependent 3' cleavage product was evident, especially with wt gRNA, as were chimeras. A faint band corresponding to a product edited by removal of three Us was occasionally observed mC₄₅ and wt gRNA (Fig. 3B, lane 3). Although other scenarios can be envisioned, it seems likely that this is the result of cleavage between U₄₄ and C₄₅, as chimeras are produced that correspond to cleavage at this site. None of the 3' cleavages described here (Fig. 3B) occurred in the absence of gRNA (data not shown). Substitution of A or G, rather than C, for the Us at the various positions in the ES also resulted in the produc-

TABLE 1. Summary results from changes in upstream nucleotides.

gRNA	pre-mRNA		
	A6U5 (mA ₄₁)	mG ₄₁	mC ₄₁
ΔG (gU ₁₉)	+/+	+/+	-/+
gC ₁₉	-/+	+/+	-/+
gG ₁₉	-/-	-/-	-/-

The relationship between the potential for (+) or not for (-) base pairing 5' to ES1 between various pre-mRNA and gRNA combinations and the production (+) or lack of production (-) of edited RNA is shown.

FIGURE 3. Effect of nucleotide substitution within the editing site. **A:** Nucleotide sequences at and near the ES1 of A6U5 and four derived substrates are shown. In each derived substrate, a single substitution (U to C) was made at a specific position as indicated in the name. The potential base pair upstream of ES1 is indicated as in Figure 1. **B:** Products of pCp 3' end labeled in vitro editing reaction containing mRNA as indicated above each lane, and either gA6[14] (WT) or gA6[14]ΔG (ΔG). Lane 1 is partial T1 digestion of A6U5 and lane 2 is a no-gRNA control containing A6U5. Input pre-mRNA (Input) and 3' fragments (3'CL) cleaved at ES1 as directed by gA6[14] (WT) or gA6[14]ΔG (ΔG) are indicated by arrows. Populations of edited RNA with one, two, three or four deleted Us (Edited) and 3' fragment-gRNA chimeras (Chimeras) are indicated by braces. **C:** Effect of nucleotide substitution on nucleotide removal from the 5' fragment. 5' end labeled in vitro editing reaction of substrates shown in **A**. Substrates were 5' end labeled with [γ -³²P]-ATP using T4 kinase as described in Materials and Methods. Otherwise, reactions are the same as in **B**. 5' cleavage fragments (5'CL) are indicated by a brace; input and edited RNA are shown as in **B**.

tion of edited RNA in which only the Us 3' of the introduced non-U were removed (data not shown). Hence, the selective removal only of Us from the 3' end of the 5' cleavage during editing suggests that the editing 3' exonuclease is specific for uridylates and thus contributes to the specificity of editing.

To directly visualize nucleotide removal from the 3' end of the 5' cleavage products, 5' end-labeled pre-mRNA was used in editing reactions along with the gRNAs described above (Fig. 3C). Incubation of A6U5 substrate RNA with gA6[14]ΔG resulted in production of a 5' cleavage fragment that corresponds to cleavage at the ES between U₄₅ and U₄₆ as well as products corresponding to fragments from which the 3' terminal one, two, three, or four Us were removed (Fig. 3C, lane 12, bottom panel). During in vitro incubation of the mC₄₂, mC₄₃, and mC₄₄ substrates with gA6[14]ΔG, products that correspond to cleavage between U₄₅ and U₄₆ were generated. In addition to the initial 5' cleavage populations, 5' cleavage products were observed from which some or all 3' terminal Us were removed. Specifically, products corresponding to those from which one, two, or three Us are removed with mC₄₂ (Fig. 3C, lane 10), one or two Us are removed with mC₄₃ (Fig. 3C, lane 8), or one U is removed with mC₄₄ (Fig. 3C, lane 6) are produced. In the case of the mC₄₅ substrate, the cleavage shifted 1 nt downstream (to between U₄₆ and G₄₇), and a 5' cleavage product from which the single 3' terminal U was removed is produced (Fig. 3C, lane 4). Upon incubation with wt gRNA, the A6U5 pre-mRNA was endonucleolytically cleaved between U₄₄ and U₄₅, and products corresponding to fragments from which one to three Us were removed are observed (Fig. 3C, lane 11). The mC₄₂ and mC₄₃ substrates are both cleaved at the same site and 1 or 2 nt were removed from mC₄₂ and 1 nt from mC₄₃ (Fig. 3C, lanes 9 and 7, respectively). The mC₄₄ substrate was not cleaved when incubated with wt gRNA (Fig. 3C, lane 5) as was the case using 3' end-labeled substrate RNA. When the mC₄₅ substrate was used, the cleavage shifted to position between U₄₆ and G₄₇, presumably due to altered base-pairing interaction that bulged the U at position 46. One U was subsequently removed from this initial cleavage product (Fig. 3C, lane 3). In summary, the 5'-labeled substrate data confirm that only Us at the 3' terminus of the 5' cleavage product are removed whether or not there is a base pair immediately upstream of the editing site.

DISCUSSION

This article provides insight into the mechanism that specifies the number of Us that are removed at a single ES during deletion editing. The ES is selected by the initial endonucleolytic cleavage, as previously shown (Seiwert et al., 1996). However, we show here that the anchor duplex alone is not invariably sufficient to direct

the endonucleolytic cleavage immediately 5' to the duplex. This suggests a more complex recognition of ESs by the endonuclease. We show that the 3' exonuclease that acts subsequent to cleavage during RNA editing is specific to Us (Fig. 3). Thus, some of the precision of deletion RNA editing reflects the specificity of this 3' exonuclease activity. In addition, this article shows that gRNA/mRNA base pairing immediately 5' to the ES is not essential for accurate deletion editing.

The 3' terminal exouridylylase (3' exoUase)

The 3' exonuclease that is associated with RNA editing removes nucleotides from the 3' terminus of the pre-mRNA 5' cleavage product as previously described (Seiwert et al., 1996; Rusché et al., 1997). We show here that this activity, in the context of full-round deletion editing, is specific for Us. The replacement of any U in the ES with A, C, or G results in edited mRNA in which only the Us that are 3' to the non-U nucleotide are removed. Consequently, we suggest a designation for this activity as the editing-associated 3' terminal exouridylylase (3' exoUase). The characteristics of the 3' exoUase other than its specificity for Us (Fig. 3; Aphasizhev & Simpson, 2001) are not yet known. The observed populations of 5' cleavage products with varying number of Us (Fig. 3C; Seiwert et al., 1996) are consistent with the distributive action of purified exoUase from *L. tarentolae* (Aphasizhev & Simpson, 2001). One report suggests that the activity that removes Us during editing is distinct from that which adds Us based on sensitivity to pyrophosphate (Cruz-Reyes & Sollner-Webb, 1996), and Us removed are released as free UMP (Rusché et al., 1997), whereas TUTase uses UTP as a substrate. Thus, U addition and removal are unlikely to be catalyzed by the same enzyme.

Base pairing 5' to the ES

It is unlikely that the 3' exoUase activity alone is sufficient for the precision of U deletion editing at a site selected for editing by endonucleolytic cleavage. Selectivity during the RNA ligation step may also contribute to the specificity of editing by gRNA-mediated alignment of the appropriately processed 5' cleavage product with the 3' cleavage product. Preferential ligation of appropriately processed 5' cleavage products has been proposed to contribute to the specificity of in vitro insertion editing (Kable et al., 1996; Igo et al., 2000). In addition, the presence of a potential upstream base pair greatly enhances the accuracy and efficiency of insertion editing (R.P. Igo, Jr., S.D. Lawson, & K. Stuart, submitted). We found that a base pair between gRNA and the pre-mRNA nucleotides immediately 5' to the ES is not necessary for in vitro deletion editing, in agreement with recently published results (Cruz-Reyes et al., 2001). This is evident, as we show here that

molecules lacking a mRNA/gRNA base pair immediately 5' to the ES are edited and hence ligated. The lack of a requirement for this base pair in ligation is also implied by the production of chimeras and partially edited RNAs in vivo, as such a base pair would not invariably be predicted. Thus, the fragments that are ligated need not be brought into proximity by such stringent base pairing with the gRNA. These data also show that molecules with any of the 4 nt on the 3' end of the 5' cleavage product can be ligated to the 3' products, whether or not its terminal nucleotide is base paired with the gRNA. Thus, the editing ligase appears to be sequence nonspecific and can ligate any 5' cleavage fragment. Presumably, the 5' fragment is held in proximity to the 3' fragment by its association with the editing complex and/or with the gRNA. Examination of preedited mRNA and cognate gRNAs shows that a base pairing is predicted just 5' of essentially all sites edited in vivo (Simpson et al., 1998). Although base pairing just 5' of the ES is not essential for ligation and editing, it may be important. Perhaps this base pairing facilitates editing of upstream sites by extending the gRNA and pre-mRNA duplex. Complete base pairing between the gRNA and pre-mRNA may not be an absolute requirement, as mismatches have been observed between gRNA and the edited mRNA sequence.

Despite the relatively indiscriminate character of the editing associated with RNA ligase, the interactions between the gRNA and the 5' region of pre-mRNA appear to be critical to editing. Some mutations that can affect this interaction ablate both deletion and insertion editing and can increase chimera production (Kable et al., 1996; S. Lawson, unpubl. results). Indeed, changes in the pre-mRNA or gRNA sequences that predict greater base pairing of gRNA to pre-mRNA 5' to the ES overcome blockages of in vitro insertion editing resulting from modification of the gRNA 3' hydroxyl (Burgess et al., 1999). Thus, it appears too simplistic for a single base pair immediately upstream of the ES to be a critical determinant for accurate editing. It seems likely that stable interactions between the 5' pre-mRNA cleavage product and the 3' region of the gRNA, the anchor duplex, and perhaps associations of the RNAs with the complex that catalyzes editing all contribute to the accuracy of Us deletion (and insertion) editing (Kabb et al., 2001). Indeed, absolute specificity at one or more steps may not be needed to result in precisely edited mRNA because incompletely edited sites may be reedited to the accurate sequence (Koslowsky et al., 1991).

ES selection

The studies presented here provide some insights into ES selection, presumably reflecting the properties of the editing-associated endoribonuclease. For example, substitutions that result in C as the first mismatched nucleotide in the preedited mRNA blocked cleavage at

that site (Fig. 3; S. Lawson, unpubl. results). Indeed, cleavage does not occur with a C immediately 5' to potential cleavage sites in several instances. Although U insertions do occur 3' of C *in vivo*, they constitute just 3% of all insertion sites observed in *T. brucei* (Burgess et al., 1999). This suggests that cleavage 3' to C is disfavored. This may reflect the specificity of the endonuclease that resembles that of *E. coli* RNase III (Zhang & Nicholson, 1997), and the nuclease that processes 18S rRNA (Venema et al., 1995).

Model for accurate editing

Our data suggest a model for precise U deletion editing that builds on the cleavage/ligation model first proposed by Blum and Simpson (1990) and confirmed by others (Cruz-Reyes & Sollner-Webb, 1996; Seiwert et al., 1996). Firstly, the site to be edited is selected by cleavage by an editing-associated endoribonuclease. The substrate specificity of this endonuclease is uncertain but it normally cleaves 3' to an unpaired pre-mRNA nucleotide that is 5' to a pre-mRNA/gRNA duplex, leaving the phosphate on the 3' cleavage product (Seiwert et al., 1996; Piller et al., 1997). However, the gRNA/mRNA base pairing flanking the ES and perhaps other structural features, including a proximal C, appear to influence the site selected for cleavage and hence editing (R.P. Igo, Jr., S.D. Lawson, & K. Stuart, submitted). Following cleavage at deletion sites, all 3' terminal Us are removed from the pre-mRNA 5' cleavage fragment by a 3' exoribonuclease that is specific for Us. Their removal is sequential, until a non-U nucleotide is encountered (Fig. 3C). At insertion sites, Us are added to the 3' terminus of the pre-mRNA 5' cleavage fragment by a terminal transferase, which is U-specific (Kable et al., 1996; N. Lewis Ernst, R.P. Igo, Jr., B. Panicucci, A.K. Panigrahi, & K. Stuart, in prep.). Addition of Us also appears to be sequential and is responsive to gRNA sequence (Byrne et al., 1996; Kable et al., 1996; Igo et al., 2000). Populations of 5' cleavage fragments that vary in numbers of Us at their 3' termini are thus generated during both deletion and insertion editing (Fig. 3C; Kable et al., 1996; Seiwert et al., 1996; Igo et al., 2000). The subset of these 5' pre-mRNA fragments that has the number of Us specified by the gRNA is preferentially ligated to the 3' fragment to produce RNA that is accurately edited at this site (Seiwert & Stuart, 1994; Cruz-Reyes & Sollner-Webb, 1996; Kable et al., 1996; Seiwert et al., 1996). Thus the endonuclease, uridylyte removal and addition, and RNA ligation steps are proposed to act in concert with each conferring a degree of specificity and act together to result in accurate editing.

Sollner-Webb and colleagues (Cruz-Reyes et al., 1998a) suggested that insertion and deletion editing may be catalyzed by different sets of enzymes, based on a differential effect of ATP concentration on cleav-

age at candidate deletion versus insertion editing sites. However, we found that reduction of ATP from 3 mM to 0.3 μ M did not affect cleavage at deletion editing sites with the substrates used here (S. Lawson, unpubl. results) and at high (3 mM) ATP concentration, cleavage at a potential insertion site is similar to that at a deletion site using the same substrate (Fig. 2, lane 9 versus lanes 8 and 10). The differences between the results in different laboratories may reflect the use of a editing complexes enriched by glycerol gradient fractionation here versus the use of ion-exchange columns. The endonuclease or endonucleases that cleave insertion and deletion sites remain to be identified.

Several aspects of our model remain unresolved. The selection of the editing site by the endonucleolytic cleavage appears complex. As we show here, a single unpaired nucleotide 5' to a duplex is not invariably an efficient endonuclease substrate. This suggests that gRNA/pre-mRNA interaction other than predicted base pairing, including interactions with the editing complex may affect the specificity of the cleavage step. This resembles the previous observation that some gRNA/pre-mRNA mismatches appear not to be edited (Koslowsky et al., 1991). It is also consistent with the proposals that sites need not be selected for editing in a precise 3' to 5' order (Koslowsky et al., 1991) and that ESs may be reedited (Abraham et al., 1988). Rereading may essentially provide a proofreading function that would eliminate imprecision in editing at any site and drive editing to the sequence specified by the gRNA because fully edited RNA would no longer be a substrate for the endoribonuclease activity (Koslowsky et al., 1991).

It is likely that the editing complex contains both U removal and U addition activities (Corell et al., 1996; Rusché et al., 1997; Cruz-Reyes et al., 1998b) but how these are organized to produce accurately edited RNA is uncertain. Perhaps, upon cleavage at an editing site, the exonuclease removes any unpaired Us on the 3' terminus of the cleavage product (Cruz-Reyes et al., 2001). During U insertion, Us added to the 5' cleavage product may be prevented from being removed by pairing with nucleotides in the gRNA (Byrne et al., 1996). Any excess added Us would then be unpaired and hence a substrate for the 3' exonuclease. Base-paired terminal Us are a poor substrate for the U-specific 3' exonuclease (Aphasizhev & Simpson, 2001; Igo et al., 2002). This suggests a substantial role for the exonuclease in specifying the number of Us in the final edited RNA at both deletion and insertion sites (Byrne et al., 1996). Nevertheless, a family of 5' cleavage products with different numbers of 3' terminal Us is generated, at least *in vitro*, although accurately edited RNA is primarily produced (Fig. 3C; Seiwert et al., 1996). Perhaps molecules that are in the process of U removal or addition are less available for ligation. Alternatively, the 5' cleavage products with the number of Us specified by

the gRNA may be preferentially ligated, perhaps simply because interactions with the gRNA and editing complex may readily bring the 3' hydroxyl and 5' phosphate of the substrate fragments into the active site of the ligase. These studies highlight how the separate activities that are present in the editing complex act in concert to generate the precise sequence of the edited mRNA, which often involves the insertion of hundreds and deletion of tens of uridylates. It will be intriguing as well as important to elucidate the characteristics of this complex and its components.

MATERIALS AND METHODS

Cell growth, mitochondrial vesicle isolation, and extract preparation

The procyclic life cycle stage of *T. brucei* strain IsTaR 1.7a (Stuart et al., 1984) was grown in SDM-79 medium (Brun & Schönenberger, 1981) to a density of $2\text{--}3 \times 10^7$ cells/mL (late logarithmic phase). Mitochondrial vesicles were isolated as described by Pollard et al. (1992). Extracts used for editing were prepared as follows. Fresh or previously frozen mitochondrial vesicles were centrifuged at $15,000 \times g$ for 15 min, lysed by the addition of $1 \times$ lysis buffer (0.5% Triton X-100; 25 mM Tris-HCl, pH 7.9; 50 mM KCl; 10 mM MgOAc; 0.5 mM DTT). They were then incubated at 4 °C for 10 min with shaking and clarified by centrifugation at $15,000 \times g$ for 5 min. Then either the 20S fraction from centrifugation of $\sim 2 \times 10^{10}$ cell equivalents in a 10–30% linear glycerol gradient (Corell et al., 1996) or the void volume fraction from a Superdex S-200 column (Pharmacia) of $\sim 5 \times 10^8$ cell equivalents was collected. The void volume fraction contains all in vitro editing activity (T. Allen & H.U. Göringer, unpubl. results).

Plasmids and PCR products

The A6short/TAG.1 (A6U5) and pgA6[14]Δ16G plasmids (Seiwert & Stuart, 1994) were used for the production of pre-mRNAs and gRNAs, respectively. All other templates for transcription were derived from these plasmids by mutagenic PCR. All synthetic oligonucleotides used in this study are shown in Table 1 and were gel purified on 12% nondenaturing polyacrylamide (19:1 w/w acrylamide:bis) gels and resuspended to approximately 20 μM. Each PCR reaction contained 20 nmol of template plasmid, 20 pmol each of 5' and 3' primers, $1 \times$ reaction buffer (10 mM Tris-HCl, pH 8.9; 50 mM KCl; 0.1% Triton X-100), 1.5 mM MgCl₂, 200 μM dNTPs, and 5 U Taq polymerase (Promega) per 100 μL reaction volume. Each PCR for the generation of template for substrate RNA production utilized the A6short/Tag.1 plasmid, the *EcoRIT7* (5') primer to add the T7 promoter, and the respective antisense (3') primers shown in Table 2. Products from the first round of PCR were separated on a 3% NuSieve (FMC) agarose gel, purified using Spin-X columns (Costar) and reamplified using the same set of primers. The resulting products from the second round were used for transcription to ensure that the parental plasmid did not contaminate the template.

Similarly two rounds of PCR were used to produce mutant templates for gRNA transcripts. The first round used pgA6[14]Δ16G (Seiwert & Stuart, 1994) as template, a 5' mutagenic oligonucleotide (see Table 1), and a common –21 reverse primer (NEB) for the 3' end. These products were purified away from the initial template using a 3% NuSieve agarose gel and used for subsequent rounds of PCR, with the same set of primers that were used in the first round. Products from the second round were digested with *DraI* (Gibco-BRL) and separated on 3% NuSieve agarose gel. DNA of the correct size was isolated from a gel slice by using the Spin-X column. Mutant versions of A6U5 are designated as mN_{##}, where N is the substituted nucleotide and ## the position of the mutation. The gRNA gA6[14] (Hinz & Göringer,

TABLE 2. Oligonucleotides used in this study.

Name	Sequence
OgG19	5'–TTCTGTAATACGACTCACTATAGGATATACTATAACTCCAGAACG–3'
OgC19	5'–TTCTGTAATACGACTCACTATAGGATATACTATAACTCCACAACG–3'
OmG41	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAAACAC–3'
OmC41	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAAAGCACA–3'
OmC42	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAAATCACA–3'
OmC43	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAGATCACA–3'
OmC44	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAGATCACA–3'
OmC45	5'–GATGCCAGGTAAGTATTCTATAACTCCAAAGATCACA–3'
EcoRIT7	5'–CGGCGGAATTCTGTAATACGACTCAC–3'
3'TAG/A6	5'–GATGCCAGGTAAGTATTC3–3'
M13(-20)	5'–GTAAAACGACGGCCAGT–3'

The oligonucleotides beginning with Om or Og prefix were used for introducing mutations in the pre-mRNA substrates and gRNAs, respectively. The other three oligonucleotides were used as the second PCR primers in the production of the templates for the RNAs as described in Materials and Methods. Nucleotides introducing mutations in RNA transcripts are in bold italics.

1999) is abbreviated as wt. Mutant versions of gA6[14]Δ16G are abbreviated as gN₁₉ to indicate the nucleotide substitution at position 19. The mutagenic oligonucleotides that were used to produce the substrate RNA and gRNAs have a similar designation with an (O) prefix.

RNA production and labeling

Guide RNA and mRNA substrates were transcribed using T7 RNA polymerase (Promega) from either plasmid or PCR product templates according to the manufacturer's recommendations. The RNA products were separated on a 9% (19:1 w/w acrylamide:bis) denaturing polyacrylamide gel containing 7 M urea and 1× TBE with 0.025% each of bromophenol blue and xylene cyanol as tracking dyes. RNA of the appropriate size was visualized by UV shadowing, eluted at room temperature from an excised gel slice by overnight incubation in elution buffer (0.5 M ammonium acetate; 2 mM EDTA; 0.1% SDS). It was then precipitated by adding 2.5 vol absolute ethanol, collected by centrifugation at 15,000 × *g*, and dissolved in water.

Approximately 20 pmol of substrate RNA were 3' end labeled with 60 μCi (3,000 Ci/mmol) [5'-³²P]pCp using T4 RNA ligase (Gibco-BRL) in 1× T4 ligase buffer (25 mM HEPES, pH 7.9; 5 mM MgCl₂; 1.6 mM DTT; 25 μM ATP), 15% DMSO and 10% glycerol. The reaction mixture was incubated overnight at 4 °C, precipitated with 2.5 vol ethanol containing 5 μg glycogen. The precipitate was washed in 70% ethanol, resuspended in 10 μL loading dye (7 M urea, 1× TBE, and 0.025% each of bromophenol blue and xylene cyanol), and run on a 9% denaturing polyacrylamide gel in 1× TBE. The 5' ends of transcripts (20 pmol) were labeled with T4 polynucleotide kinase. First, the 5' end was dephosphorylated with 1 U calf-intestinal alkaline phosphatase in 50 mM Tris-HCl, pH 7.9; 100 mM NaCl; 10 mM MgCl₂; 1 mM DTT at 37 °C for 1 h. The sample was then extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated at -20 °C for 1 h after the addition of 0.1 vol 3 M NaOAc (pH 5.2) and 2.5 vol absolute ethanol. The RNA was collected by centrifugation for 30 min at 4 °C, the pellet was washed with 70% ethanol, dried, and dissolved in water. Dephosphorylated RNA was 5' end labeled with 60 μCi (3,000 Ci/mmol) [γ-³²P]ATP using 10 U T4 polynucleotide kinase (New England Biolabs) in 1× buffer (70 mM Tris-HCl, pH 7.6; 10 mM MgCl₂; 5 mM DTT) at 37 °C for 1 h. Following the reaction, samples were treated as described above for 3' labeled RNA. RNA of the appropriate size was visualized by autoradiography, excised, eluted, and precipitated as described above. The RNA was dissolved in distilled water to a final concentration of 0.25 μM. The mutations that were introduced into each substrate pre-mRNA or gRNA were confirmed by sequencing of the labeled RNA using a commercially available kit (Pharmacia).

RNA editing reaction

In vitro editing reactions were performed essentially as described by Seiwert and Stuart (1994) in a final volume of 30 μL, using 0.25 pmol of labeled substrate mRNA, 2.5 pmol gRNA, and 10–15 μL mitochondrial extract (~1–5 μg protein) from a 20S glycerol gradient or Superdex S-200 void volume fraction. Samples were extracted with phenol/

chloroform/isoamyl alcohol (25:24:1) after incubation, precipitated, and dissolved in loading dye. Products were separated on a 9% polyacrylamide gel and visualized on the dried gel by autoradiography using X-ray film or a Storm PhosphorImager (Molecular Dynamics).

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