In vitro uridine insertion RNA editing mediated by *cis*-acting guide RNAs

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ABSTRACT

Uridine (U) insertion/deletion editing of mitochondrial mRNAs in kinetoplastid protozoa is a posttranscriptional process mediated by guide RNAs (gRNAs). The gRNAs direct the precise insertion and deletion of Us by a cleavage–ligation mechanism involving base pairing. We show that a cognate gRNA *in cis* at the 3′ end of a preedited NADH dehydrogenase 7 (ND7) mRNA substrate can direct U insertions at editing site 1 when incubated with a mitochondrial lysate from *Leishmania tarentolae*. The efficiency of gRNA-dependent U insertion mediated by a *cis*-acting gRNA is greater on a molar basis than that for a *trans*-acting gRNA, as expected for a unimolecular gRNA:mRNA interaction. Blocking the 3′ end of a *cis*-acting gRNA lacking a 3′ oligo[U] tail has no effect on gRNA-dependent U insertions, nor does providing the gRNA *in cis* upstream of the mRNA, confirming the previous observation that the terminal 2′- and 3′-hydroxyls of the gRNA are not involved in U insertion activity. These results also establish that the oligo[U] tail is not required for U insertion *in vitro*. Increasing the extent of base pairing between the 3′ end of the gRNA and the 5′ end of the mRNA significantly increases *in vitro* gRNA-dependent U insertion at site 1, presumably by maintaining the mRNA 5′ cleavage fragment within the editing complex. We speculate that, *in vivo*, protein:RNA and/or protein:protein interactions may be responsible for maintaining the mRNA 5′ cleavage fragment in close proximity to the mRNA 3′ cleavage fragment, and that such interactions may be rate limiting *in vitro*.

Keywords: guide RNA; *Leishmania*; RNA editing; uridine insertion

INTRODUCTION

RNA editing in kinetoplastid protozoa involves the post-transcriptional insertion and deletion of uridine (U) residues within mitochondrial mRNAs (Alfonzo et al., 1997; Stuart et al., 1997). The mitochondrial genome of *Leishmania tarentolae* is a catenated network of 20–50 maxicircles of approximately 30,000 bp and several hundred minicircles of approximately 900 bp. Twelve of the eighteen structural genes of the maxicircle component of the mitochondrial genome of *L. tarentolae* are cryptogenes, the transcripts of which are altered by RNA editing. These editing events can create initiation and termination codons, overcome reading frame shifts, and in the cases of pan-edited transcripts, create entire reading frames in which the posttranscriptionally inserted Us account for more than half of the nucleotide content of the transcript.

The insertion and deletion of Us at editing sites is directed by short mitochondrial transcripts termed guide RNAs (gRNAs; Blum et al., 1990). Some gRNA genes are located in the maxicircle DNA, but the majority are encoded in the minicircles (Sturm & Simpson, 1990a, 1991; Pollard et al., 1990; Simpson, 1997). The formation of an anchor duplex between the 5′ end of the gRNA and the preedited mRNA just downstream of an editing site represents the initial event of editing. With only one exception, gRNAs *in vivo* are provided *in trans*; the gRNA for the frameshift editing of the cytochrome oxidase subunit II (COII) mRNA is located *in cis* at the 3′ end of the preedited transcript (Blum et al., 1990). The gRNAs contain guiding adenosine (A) and guanosine (G) nucleotides, which direct the precise insertion and deletion of Us at editing sites. Editing site selection generally proceeds 3′ to 5′ both within a single editing block (Sturm & Simpson, 1990b), and within an editing domain comprised of multiple overlapping gRNAs (Maslov & Simpson, 1992).

Data from *in vitro* editing systems (Seiwert & Stuart, 1994; Frech et al., 1995; Byrne et al., 1996; Cruz-

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Reyes & Sollner-Webb, 1996; Frech & Simpson, 1996; Kable et al., 1996; Seiwert et al., 1996), as well as the analysis of individual enzymatic activities (Bakalara et al., 1989; Harris et al., 1992; Simpson et al., 1992; Sabatini & Hajduk, 1995), have given strong support for a protein-mediated cleavage–ligation mechanism of RNA editing known as the enzyme cascade model (Blum et al., 1990). This model predicts that the initial enzymatic step of RNA editing is a gRNA-dependent endonucleolytic cleavage of the preedited mRNA at the 3' most editing site to produce mRNA 5' and 3' cleavage fragments. Cleavage is followed by processing of the mRNA 5' fragment by addition or deletion of Us at the 3' end as directed by base pairing with the cognate gRNA. Religation of the mRNA cleavage fragments yields an mRNA edited at a given site.

An *in vitro* editing system has been previously established for U deletion at site 1 (Seiwert & Stuart, 1994) and U insertion at site 2 (Kable et al., 1996) of the *Trypanosoma brucei* ATPase 6 (A6) mRNA using the 20S fraction of a mitochondrial lysate. The efficiency of this *in vitro* editing system was approximately 1–5%, which was sufficient to allow a direct visualization of editing intermediates. These studies showed that the precise cleavage of the substrate mRNA 3' of the Us that are to be deleted is directed to occur at the first mismatch between the mRNA and the cognate gRNA provided *in trans* (Seiwert et al., 1996). For deletion, Us are then sequentially removed from the 3' end of the mRNA 5' cleavage fragment. A precise cleavage occurs at site 2 if a substrate that is edited in site 1 is provided. For insertion at site 2, U residues are transferred from UTP to the 3' end of the mRNA 5' cleavage fragment, and these inserted Us base pair with the guiding nucleotides in the gRNA (Kable et al., 1996). The adenylate nucleotide requirements for cleavage at the U deletion site and the U insertion site differ, raising the possibility that these represent different enzymatic pathways (Cruz-Reyes et al., 1998). Chimeric molecules with the 3' end of the gRNA linked to the 5' end of the mRNA 3' cleavage fragment occur, but appear to represent nonproductive intermediates of the reaction, as chimera formation can be blocked without greatly affecting the production of the fully edited species (Seiwert et al., 1996).

An *in vitro* U insertion editing system has also been established for domain II of the NADH dehydrogenase 7 (ND7) mRNA of *L. tarentolae* (Byrne et al., 1996). In this case, the efficiency was too low to permit direct visualization of intermediates, and a PCR based primer extension assay was employed. Both gRNA-independent and gRNA-dependent U insertion activities were detected using a crude mitochondrial lysate or a 20S fraction. In the absence of a cognate gRNA, mRNA substrates were modified by the insertion of approximately 1–13 Us into the first editing site, with the extent of this activity being dependent on the pres-

ence of an extended 5' and 3' sequence in the mRNA. Upon the addition of cognate gRNA provided *in trans*, the directed insertion of a specific number of Us at editing site 1 was observed in addition to the background insertion of a ladder of Us. Addition of a gRNA with no guiding nucleotides for the first editing site decreased the extent of gRNA-independent U insertions. These observations strongly support a modified cleavage–ligation model, which states that after cleavage of the preedited mRNA at an editing site, multiple Us are added to the 3' end of the mRNA 5' cleavage fragment in an untemplated manner, and then excess Us that are not base paired to guiding nucleotides are removed by a 3'–5' exonuclease (Byrne et al., 1996; Alfonzo et al., 1997).

The role of the nonencoded 3' oligo[U] tail of the gRNAs is unknown, although it has been proposed to form a 3' duplex with the preedited region (Blum & Simpson, 1990; Arts et al., 1995), thereby stabilizing the gRNA–mRNA interaction and also maintaining the mRNA 5' cleavage fragment within the editing complex. In the *T. brucei* U deletion assay (Seiwert et al., 1996), removal of the 3' oligo[U] tail had no effect on cleavage of the preedited mRNA, but inhibited the production of the edited product. Mutation of the 3' end of the gRNA to increase its base pairing interaction with the mRNA 5' cleavage fragment somewhat decreased the formation of edited product. Blocking of the 3' terminus of the gRNA by periodation completely inhibited U deletion editing in the *T. brucei* system (Seiwert et al., 1996), but had no effect on U insertion editing in the *L. tarentolae* system (Byrne et al., 1996).

A requirement to provide an excess of cognate gRNA *in trans* with the preedited mRNA substrate is characteristic of both the *T. brucei* and *L. tarentolae* *in vitro* editing systems (Seiwert & Stuart, 1994; Byrne et al., 1996; Kable et al., 1996; Seiwert et al., 1996). Based on the *in vivo* existence of the COII mRNA–gRNA transcript, we have examined the effect of *cis*-acting gRNAs on U insertion editing at site 1 of an *L. tarentolae* ND7 mRNA substrate. We show that providing a cognate *cis*-acting gRNA increases the efficiency of the gRNA–mRNA interaction and allows informative manipulations of the editing reaction to be performed.

RESULTS

U insertion into the first editing site of two ND7 mRNA substrates can be directed by 3' *cis*-acting gRNAs

We examined, by the indirect primer extension assay diagrammed in Figure 1, the ability of a gRNA to direct U insertion *in vitro* when placed *in cis* with the 165-nt anchor-mutated ND7.2x mRNA substrate, which is diagrammed in Figure 2A. The *cis*-acting gRNA contained

FIGURE 1. Diagram of the indirect primer extension assay developed by Byrne et al. (1996) to detect U insertions in the first editing site of editing domain II in mutated anchor (x) ND7 mRNA substrates. The preedited region (PER) of the RNA is indicated by a bracket, and shown are +3 Us inserted into editing site 1. Mutated anchor sequence is indicated by thicker lines, the position of each oligonucleotide is noted, and the lighter portions of oligonucleotide S-1529 and the sense DNA indicate sequences used for normalization.

compensatory mutations in the anchor sequence to preserve base pairing with the mRNA (Byrne et al., 1996). The results in Figure 3A show that the *cis*-acting gRNA is capable of directing U insertions *in vitro* at levels comparable to those directed by a tenfold molar excess of *trans*-acting gRNA. Transcripts with gRNAs guiding +3, +4, and +7 U insertions were used, and showed comparable efficiencies. Use of a *cis*-acting gND7[+3], which has a wild-type anchor sequence, resulted in the loss of gRNA-directed U insertions and the appearance of a background ladder of gRNA-independent U insertions (Fig. 3A, lane 11), as obtained in the absence of added gRNA (Fig. 3A, lane 2). This type of U insertion activity has been observed previously with the ND7.2x mRNA (Byrne et al., 1996), and a cytochrome *b* (CYb) mRNA substrate (Peris et al., 1994; Frech et al., 1995), and was attributed to mRNA foldback secondary structure mimicking the gRNA-mRNA anchor duplex (Connell et al., 1997).

The use of a *cis*-acting gRNA with no guiding nucleotides, ND7.2x/gND7x[+0], yielded only the artifact band at +1 with no additional U insertions (Fig. 3A, lane 7). An identical result was obtained with a tenfold molar excess of the nonguiding cognate gND7x[+0] *in trans* (Fig. 3A, lane 3).

To eliminate the possibility that the anchor sequences of the mRNA and gRNA portions of the *cis* transcripts could hybridize in an intermolecular fashion to act *in*

trans between separate RNAs, a quantitative comparison of the extent of U insertions directed by a gRNA *in cis* or by increasing amounts of the same gRNA *in trans* was performed. As shown in Figure 3B, the extent of U insertions in the ND7.2x mRNA directed by a *cis*-acting gND7x[+3] (Fig. 3B, lane 1) is approximately equivalent to the extent of U insertions directed by a 7.5-fold molar excess of the same gRNA provided *in trans* (Fig. 3B, lane 7). This experiment indicates that the gRNA-dependent U insertions obtained with the transcripts with gRNAs *in cis* are primarily due to intramolecular, and not intermolecular, mRNA:gRNA interactions. Additional evidence for this was obtained by providing an ND7.2x/gND7x[+3] transcript with *trans*-acting gND7x[+4] in a 1:2 molar ratio and observing a predominant gRNA-dependent insertion of +3 Us (Fig. 5, lane 4).

Similar experiments were performed using the 65-nt anchor-mutated ND7.4x mRNA substrate with a cognate gRNA *in cis*, which is diagramed in Figure 2B. As in the ND7.2x situation, a gRNA-independent ladder of U insertions was observed with a transcript that lacked the gRNA *in cis* (Fig. 4A, lane 2) or a transcript that contained a *cis*-acting gRNA that had a wild-type anchor sequence (Fig. 4A, lane 11). It should be noted that the level of gRNA-independent U insertion activity with the ND7.4x substrate in this study is greater than reported previously, which may be due to the use of

FIGURE 2. Schematic diagrams of the *cis*-acting gRNA transcripts used in this study. Each diagram is shown approximately to scale. 5' and 3' ends are labeled; filled segments represent gRNA sequence; unfilled segments represent mRNA sequence; thicker portions represent anchor sequence; vertical arrows denote editing site 1. Each top panel is a linear depiction of the transcript in which mRNA and gRNA regions are labeled. Each lower panel shows the proposed folding to form an intramolecular anchor duplex. Nucleotide rulers indicate approximate nucleotide position. **A:** ND7.2x/gND7x, a 165-nt ND7 mRNA with a *cis*-acting gRNA at the 3' end. **B:** ND7.4x/gND7x, the 65-nt ND7 mRNA with a *cis*-acting gRNA at the 3' end. **C:** gND7x/ND7.4x, the 65-nt ND7 mRNA with a *cis*-acting gRNA at the 5' end.

fresh mitochondria in these experiments as compared to the use of frozen mitochondria in the previous work.

The use of a substrate with a *cis*- or *trans*-acting gND7x[+0], which has no guiding nucleotides for site 1, again yielded a total absence of the ladder of gRNA-independent U insertions (Fig. 4A, lanes 3 and 7).

As in the case of the ND7.2x substrate, the extent of +3, +4, and +7 gRNA-dependent U insertions mediated by the *cis*-acting gRNAs was approximately equivalent to the extent of insertions mediated by a tenfold excess of *trans*-acting gRNAs. This was shown quantitatively in the experiment in Figure 4B, in which the effect of increasing amounts of *trans*-acting gND7x[+3] was assayed. In addition, providing the *cis*-acting ND7.4x/gND7x[+3] transcript with *trans*-acting gND7x[+4] at a 1:2 molar ratio yielded a predominant +3 U insertion band in the extension assay (Fig. 5, lane 8). These results provide additional evidence for an intramolec-

ular reaction of the ND7.4x mRNA and cognate *cis*-acting gRNA as opposed to an intermolecular reaction.

U insertions directed by a 3' *cis*-acting gRNA are not dependent on the terminal 2'- and 3'-hydroxyls of the gRNA

In the *L. tarentolae* system, blocking the 3' terminus of a *trans*-acting gRNA by periodation has no effect on the ability to guide U insertions *in vitro* (Byrne et al., 1996). We have assayed the role of the terminal 2'- and 3'-hydroxyls of the ND7.4x/gND7x[+3] (Fig. 2B) transcript. In these experiments, approximately 94% of the transcripts remained blocked after incubation with mitochondrial lysate (data not shown). The *in vitro* gRNA-dependent U insertion directed by a *cis*-acting gRNA is not significantly affected by periodate treatment (Fig. 6, lanes 5 and 6). As described above, the relative extent of gRNA-dependent U insertion ob-

FIGURE 3. In vitro U insertion with the 165-nt ND7.2x mRNA. Transcripts were incubated under editing conditions and assayed for the insertion of Us into editing site 1. Guiding nts for site 1 indicates the number of site 1 guiding nucleotides in the gRNA; a dash indicates no gRNA was added; wta indicates a gRNA with a noncognate (wild-type) anchor. **A:** Lane 1: no lysate control; lane 2: no gRNA control; lanes 3–6: a tenfold molar excess of gRNA was used; lanes 7–10: transcripts with cognate *cis*-acting gRNAs; lane 11: transcript with noncognate gRNA. **B:** Comparison of U insertion with *cis*- and *trans*-acting gRNA. Lane 1: ND7.2x/gND7x[+3]; lanes 2–8: 3 pmol of ND7.2x mRNA was incubated with increasing amounts of gRNA, as indicated by pmols *trans* gND7x[+3]. Each column in the bar graph below corresponds to the level of +3 U insertion from each lane.

FIGURE 4. In vitro U insertion with the 65-nt ND7.4x mRNA (as for Fig. 3 above). **A:** Lane 1: no lysate; lane 2: no gRNA; lanes 3–6: a tenfold molar excess of gRNA; lanes 7–10: transcripts with cognate *cis*-acting gRNAs; lane 11: transcript with noncognate *cis*-acting gRNA. **B:** Comparison of U insertion with *cis*- and *trans*-acting gRNA. Lane 1: ND7.4x/gND7x[+3]; lanes 2–8: 3 pmol of ND7.4x mRNA was incubated with increasing amounts of gRNA, as indicated by pmols *trans* gND7x[+3]. The bar graph below shows the level of gRNA-dependent U insertion from each lane.

served with the *cis*-acting gRNA is comparable to that seen when the same gRNA is provided at a tenfold molar excess *in trans* (Fig. 6, lanes 3 and 4), regardless of whether or not the 3' ends are blocked by periodation.

The ND7.4x/gND7x[+3] substrate used in these experiments lacked a transcribed 3' oligo[U] tail, and the periodate treatment of the 3' end prevents the addition of Us during the incubation by the terminal uridylyl transferase (TUTase) in the lysate. Therefore, these results

FIGURE 5. Competition between a *cis*-acting gND7x[+3] and a twofold molar excess of *trans*-acting gND7x[+4]. The ND7.2x or ND7.4x mRNA (as indicated) was incubated under editing conditions with no gRNA (lanes 1 and 5), with only a *cis*-acting gND7x[+3] gRNA (lanes 2 and 6), with only a twofold molar excess of gND7x[+4] gRNAs (lanes 3 and 7), or with both a *cis*-acting gND7x[+3] and a twofold molar excess of gND7x[+4] gRNAs. The bar graph below shows the level U insertion from each lane, with filled bars corresponding to +3 U insertion, and striped bars corresponding to +4 U insertion.

also provide evidence that the 3' oligo[U] tail is not required for gRNA-dependent U insertions *in vitro*.

Stabilizing the interaction between the 3' end of the gRNA and the mRNA enhances *in vitro* U insertion activity

The 3' end of the gND7x[+3] gRNA was mutated to allow a 27-bp duplex between the gRNA and the 5' end of the ND7.4x mRNA, as shown in Figure 7. The effect of using this stabilized gRNA, either as a *cis*- or *trans*-acting gRNA, on gRNA-dependent U insertions was examined. The results in Figure 6 show that use of the 3'-end-stabilized gRNA in the ND7.4x/gND7x[+3] transcript yielded an enhancement of gRNA-dependent U insertions (Fig. 6, lanes 9 and 10) approximately sevenfold over that obtained with the nonstabilized substrate (Fig. 6, lanes 5 and 6); this level of enhancement was approximately equivalent to the enhancement seen with a tenfold molar excess of the same gRNA provided *in trans* (Fig. 6, lanes 7 and 8). This U insertion activity is

not affected by blocking the 3' end by periodation (Fig. 6, lanes 8, 10). It should be noted that there is also an enhancement of the primer +1 artifact band, which is also seen in control assays using an mRNA substrate that has either +3 or +7 Us in site 1 (data not shown).

The ND7.4x mRNA substrate with a 3' *cis*-acting gRNA remains intact after *in vitro* U insertions at editing site 1

In the primer extension assay, the initial reverse transcription step employs a primer complementary to the mutated anchor sequence of the mRNA (Fig. 1). To determine whether the entire substrate molecule remains intact after U insertion, a primer was used for the initial reverse transcription step of the extension assay with the ND7.4x/gND7x[+3] transcript that is complementary to the gRNA portion (oligonucleotide S-2166 in Fig. 2B). The data in Figure 8 show that use of this primer detects the same extent of gRNA-dependent U

FIGURE 6. Effect of alterations of the 3' end of the gRNA on U insertion. ND7.4x substrate mRNA was reacted with various *trans*- and *cis*-acting gRNAs (as indicated), with and without periodate treatment as indicated by periodation: + or -. Lane 1: no lysate; lane 2: no gRNA; lane 3: *trans*-acting gRNA; lane 4: periodate treated *trans*-acting gRNA; lane 5: *cis*-acting gRNA; lane 6: periodate treated *cis*-acting gRNA; lane 7: *trans*-acting gRNA with stable 3' end; lane 8: periodate treated *trans*-acting gRNA with stable 3' end; lane 9: *cis*-acting gRNA with stable 3' end; lane 10: periodate treated *cis*-acting gRNA with stable 3' end. The bar graph below shows to the level of +3 U insertion from each lane. Lanes 11–14 are a shorter exposure of lanes 7–10, to show the specificity of the gRNA-dependent +3 U insertion.

insertion at editing site 1 as use of the mRNA anchor-specific primer, indicating that the mRNA/gRNA substrate remains intact after the editing at site 1.

U insertions at editing site 1 can be directed by a *cis*-acting gRNA 5' of the mRNA

The experiments described above indicate that gRNAs can direct U insertions when placed *in cis* at the 3' end of a preedited mRNA. A substrate was constructed with a *cis*-acting cognate gRNA at the 5' end of the 65-nt preedited ND7.4x substrate RNA. As diagramed in Figure 2C, the gND7x[+7]/ND7.4x substrate could theoretically fold to produce an anchor duplex and present the preedited sequence to the editing machinery. As shown (Fig. 9, lane 4), use of this 5' *cis*-acting gRNA led to a U insertion ladder with a predominant band at +7 Us. The extent of +7 U insertions is approximately threefold greater than when the same gRNA is provided at a tenfold molar excess *in trans* (Fig. 9, lane 3); in addition, the extent of the background ladder of mis-

edited U insertions is also increased relative to both the identical gRNA provided *in trans* or *in cis* at the 3' end of the molecule.

To examine whether the substrate molecule remains intact after editing, a 5' primer (S-3020 in Fig. 2C) specific to the upstream gRNA sequence was used for the asymmetric PCR step of the primer extension assay. This selects for the amplification of intact molecules for the subsequent primer extension analysis of U insertions into editing site 1. This assay yielded the same extent of U insertion (Fig. 9, lane 5) as when a primer specific for the mRNA portion of the molecule, as in the original assay protocol, was used with either *trans*- or *cis*-acting gRNAs (Fig. 9, lanes 3 and 4).

DISCUSSION

Using an RT-PCR based primer extension assay, we have shown that a *cis*-acting cognate gRNA located either 3' or 5' of a preedited mRNA sequence can mediate U insertion editing *in vitro* using a mitochon-

FIGURE 7. Anchor hybridization of the mutated anchor transcripts, and the gRNA with the stabilized 3' end. Only the relevant portion of the mRNA is shown; the black arrows indicate editing site 1; the three guiding nucleotides for editing site 1 are boxed in the gRNA sequences. **A:** The mutated anchor mRNA:gRNA pair, showing hybridization of the anchor duplex and a posttranscriptionally added 3' oligo[U] tail. **B:** The interaction between the mRNA and a gRNA (gND7x[+3]) that has been modified to create base pairing between the 3' end of the gRNA and the mRNA 5' cleavage fragment. The substituted bases in the gRNA sequence that form the stable 3' end are in bold letters.

drial lysate from *L. tarentolae*. The gRNA undergoes fold-back hybridization to the mRNA to form an anchor duplex, and the gRNA-dependent U insertions occur in editing site 1, which is 3' of the first mismatched nucleotide. We assume that the formation of the anchor duplex initiates the editing reaction, and in the case of the *cis*-acting gRNA, this is an intramolecular reaction and thus it obeys zero order kinetics. Consistent with this, we observed an approximately tenfold greater efficiency of gRNA-directed U insertions with the gRNA *in cis* as compared to the same gRNA *in trans*. Other characteristics of the *in vitro* editing reaction appear to be similar to the reaction mediated by a *trans*-acting gRNA, that is, the background ladder of U insertions with a predominant gRNA-dependent band, the absence of U insertions with a cognate gRNA lacking guiding nucleotides for the first editing site, and the presence of a ladder of U insertions with a gRNA with a noncomplementary anchor sequence.

The only situation *in vivo* in which the gRNA is *in cis* with the preedited mRNA is the COII gene. In all kinetoplastid species examined, there is a short sequence located at the 3' end of the mRNA transcript that could function to guide the internal editing of the COII mRNA by foldback (Blum et al., 1990; Van der Spek et al., 1991; Kim et al., 1994). Evidence for the existence of

this anchor duplex is provided by the observation of compensatory mutations in the mRNA and putative gRNA that preserve base pairing in different species. In all other cases, the necessary gRNAs are provided *in trans*. Our *in vitro* results support the hypothesis that the COII 3' sequence is a *cis*-acting gRNA *in vivo* by showing that a gRNA:mRNA anchor duplex that is effective in supporting *in vitro* editing can be formed when the two sequences are separated by over 100 nt.

Our results confirm and extend the evidence that the terminal 2'- and 3'-hydroxyls of the gRNA are not required for U insertion editing. This was shown both by blocking the 3' end of the 3' *cis*-acting gRNA by periodation, and also by placing the gRNA *in cis* 5' of the mRNA. Our previous attempts to block the 3' ends of *trans*-acting gRNAs by periodate treatment showed that approximately 30% of the gRNAs lost the block upon exposure to lysate (Byrne et al., 1996), presumably as a result of 3'-end degradation by nuclease activity in the lysate. In the current experiments, we found that only approximately 6% of the molecules with *cis*-acting gRNAs were unblocked after exposure to lysate. The situation in the *T. brucei* A6 system appears different in that blocking the 3' end of the gRNA by periodation was reported to prevent editing *in vitro* (Seiwert et al., 1996).

FIGURE 8. The ND7.4x/gND7x[+3] transcript is intact after U insertion *in vitro*. The ND7.4x substrate was assayed for U insertion mediated by *trans*- or *cis*-acting gND7x[+3]. The transcript with the *cis*-acting gRNA was assayed for U insertions into editing site 1 after reverse transcription of either only the mRNA portion of the transcript (as per the original assay protocol), or reverse transcription of the entire transcript length. Lane 1: no lysate; lane 2: no gRNA; lane 3: *trans*-acting gRNA; lane 4: ND7.4x/gND7x[+3] assayed by original protocol; lane 5: ND7.4x/gND7x[+3] assayed using reverse transcription of the entire transcript length. The bar graph below shows to the level of +3 U insertion from each lane.

We showed previously that a short intramolecular duplex created by the foldback of the 5' and 3' ends of a CYb mRNA substrate resulted in gRNA-independent U insertions into the preedited region (Peris et al., 1994; Frech et al., 1995; Connell et al., 1997). In the experiments reported in this article, the anchor duplex formed by foldback of the 3' or 5' *cis*-acting gRNA resulted in both gRNA-directed U insertion and a background insertion of a ladder of Us. We speculate that double-strand RNA-binding proteins that recognize a short duplex RNA may be involved with initiation of the editing reaction in both systems, but this remains to be investigated.

The use of *cis*-acting gRNAs afforded the opportunity to examine whether or not preedited mRNAs remain attached to the gRNA after U insertion *in vitro*. The 3' and 5' *cis*-acting gRNAs were used as 3' and 5' tags during the reverse transcription and asymmetric PCR stages of the assay, respectively, thus allowing the analysis of molecules that remained intact after U insertion *in vitro*. Our results indicate that endonucle-

FIGURE 9. gRNA-dependent U insertion mediated by a 5' *cis*-acting gRNA. The ND7.4x substrate was assayed for U insertion mediated by gND7x[+7] either *in trans* or *in cis* at the 5' end of the transcript. The gND7x[+7]/ND7.4x transcript was assayed for U insertions into editing site 1 after asymmetric PCR amplification of either only the mRNA portion of the transcript (as in the original assay protocol) or amplification of the entire transcript length. Lane 1: no lysate; lane 2: no gRNA; lane 3: *trans*-acting gND7x[+7]; lane 4: gND7x[+7]/ND7.4x assayed by original protocol; lane 5: gND7x[+7]/ND7.4x assayed using asymmetric PCR amplification of the entire transcript length. The bar graph below shows the normalized levels of +7 U insertion for each lane.

olytic cleavage of RNA during U insertion *in vitro* is specific for the editing site. We did not see a change in the amount of U insertion that would indicate other cleavage events that would separate the preedited mRNA from the *cis*-acting gRNA.

The existence of a nonencoded 3' oligo[U] tail on the gRNAs *in vivo* still represents an enigma. Blum and Simpson (1990) initially proposed a role for the oligo[U] tail in hybridizing to the purine-rich preedited sequence and maintaining the mRNA 5' cleavage fragment within the editing complex prior to religation, similar to the proposed role of the conserved U residues of the U5 snRNA that align RNAs during splicing (Sharp, 1994). In an unblocked substrate it is difficult to analyze the role of the 3' oligo[U] tail because Us are added to the

3' end by the mitochondrial TUTase during the incubation. Using a 3' blocked substrate, we showed in this paper that the presence of an oligo[U] tail on a gRNA is not required for U insertion editing in vitro at a specific site. However, the initial suggestion that some mechanism is required to prevent the mRNA 5' cleavage fragment from leaving the editing complex prior to religation is still valid, and we showed that stabilization of the interaction between the mRNA 5' cleavage fragment and the 3' end of the gRNA by formation of a stable duplex does dramatically increase the efficiency of gRNA-dependent U insertions.

It should also be noted that the gRNA:mRNA anchor duplex formed by the 5' *cis*-acting gRNA would also serve to maintain the mRNA 5' cleavage fragment within an editing complex, which may explain the enhanced U insertion observed with this RNA. The 5' *cis*-acting gRNA anchor duplex would not, however, necessarily serve to accurately arrange the mRNA 5' cleavage fragment for religation, which may account for the greater amount of misediting seen with this RNA.

Our proposition that efficient U insertion involves an interaction between the gRNA and the mRNA 5' cleavage fragment is not necessarily inconsistent with our results that show the gRNA oligo[U] tail to be dispensable for editing in vitro. Retention of the mRNA 5' cleavage fragment in vivo may involve protein:RNA and/or protein:protein interactions that cannot be reproduced efficiently in vitro, possibly involving identified oligo[U] (Leegwater et al., 1995; Bringaud et al., 1997; Vanhamme et al., 1998) or gRNA binding proteins (Koller et al., 1997). The enhanced in vitro U insertion directed by a gRNA with a stable 3' end may therefore be compared to the facilitation of splicing of a group I intron by a splicing factor that can be by-passed in vitro by alteration of reaction conditions (Weeks & Cech, 1995).

The ability to manipulate editing with an mRNA with a *cis*-acting gRNA should prove useful when a mitochondrial transient transfection or stable transformation system becomes available. Use of such constructs may allow a detailed analysis of the *cis*-acting elements of RNA editing to be performed.

MATERIALS AND METHODS

Transcript nomenclature

The ND7.2x substrate is a T7 transcript that represents the first 165 nt of the *L. tarentolae* ND7 mRNA; the x indicates the presence of a mutated anchor sequence (Byrne et al., 1996). The ND7.4x substrate is a 65-nt transcript identical to ND7.2x but it has a 100-nt 3' truncation. Guide RNAs are indicated as gND7x[+n], where the g indicates a guide RNA sequence, ND7 indicates the editing of the ND7 mRNA, the x indicates the gRNA has a mutated anchor sequence complementary to the mutated mRNA anchor sequence, and the [+n] indicates the number of guiding nucleotides for editing site 1.

Transcripts with *cis*-acting gRNA sequences are indicated as in the following example: ND7.4x/gND7x[+3] indicates a transcript composed of the 65-nt ND7 mRNA substrate at the 5' end and a *cis*-acting gRNA (with three guiding nucleotides for editing site 1) at the 3' end, with both the mRNA and gRNA having mutated anchor sequences. It is important to note that none of the transcripts used in this study had 3' oligo[U] tails added during in vitro transcription. The 3' oligo[U] tails are added during lysate incubation by endogenous TUTase activity (Byrne et al., 1996), except when the RNAs are treated with sodium *m*-periodate, which blocks the 3' end and therefore prevents oligo[U] tail addition.

PCR amplification and cloning of transcription templates

Templates for T7 transcription of gRNAs and mRNAs were produced by PCR using *Taq* DNA polymerase (Promega). To amplify gRNAs, S-2172 and S-2166 were used as primers for PCR and the appropriate oligonucleotide (S-2010, S-2009, S-2088, or S-1855) was used as a PCR template. The S-2919 oligonucleotide was used to amplify the template for transcription of the gRNA with a stable 3' end (shown in Fig. 6B). To amplify a template for transcription of the 165-nt mRNA substrate, oligos S-2172 and S-245 were used as primers and an ND7.2x PCR product with a T7 promoter (kindly provided by Elaine Byrne) was used as a template. To amplify a template for transcription of the 65-nt mRNA substrate, oligos S-2172 and S-1679 were used as primers and an ND7.4x PCR product with a T7 promoter (kindly provided by Elaine Byrne) was used as a template.

A general procedure for cloning templates for in vitro transcription was as follows, using standard molecular biology techniques (Sambrook et al., 1989). Two micrograms of pUC19 plasmid DNA were digested to completion with *Sap*I (New England Biolabs); the ends of the digested vector were blunted by treatment with Mung Bean Nuclease (New England Biolabs) and then treated with CIAP (Gibco-BRL) to prevent nonrecombinant self-ligation of the vector during subsequent cloning steps. Inserts for plasmids were produced by PCR (see above). PCR reactions were extracted with phenol and precipitated with ethanol, then treated with T4 DNA polymerase (Gibco-BRL) to create blunt ends. The blunt-ended PCR products were then treated with T4 polynucleotide kinase (Gibco-BRL) to provide an insert for ligation into the above vector. Ligation reactions were performed using T4 DNA ligase (Gibco-BRL) and transformed into XL1-blue *Escherichia coli* (Stratagene). Plasmid DNA from potential clones was isolated and sequenced on both strands using Sequenase Version 2.0 (United States Biochemical) to determine the orientation and nucleotide sequence of the insert and the integrity of the *Sap*I site and the remaining vector surrounding the site. Addition of gRNA sequence to mRNA template plasmids, or mRNA sequence to gRNA template plasmids was performed by the same procedure.

In vitro RNA production

RNAs were in vitro transcribed from linearized plasmid templates using T7 RNA polymerase according to established methods (Cunningham & Ofengand, 1990). Transcription re-

actions were extracted with phenol and precipitated with ethanol, then purified from polyacrylamide-8 M urea gels.

Sodium *m*-periodate treatment of RNA and determination of 3' end blocking

Periodation of RNA was performed as described previously (Goringer et al., 1984). Three hundred picomoles of RNA were reacted at 4 °C in the dark in a 300- μ L reaction with 100 mM sodium acetate (pH 4.5) and 0.1 mM sodium *m*-periodate. RNAs were then collected using the RNaid Kit (BIO 101) and further purified from an 8% polyacrylamide-8 M urea gel.

To determine the amount of 3' end block that was maintained after exposure of the RNA to lysate, the efficiency of RNA labeling by TUTase activity was measured. RNAs were incubated with mitochondrial lysate in the presence of [α -³²P]UTP under editing conditions (see below), then extracted with phenol and separated on an 8% polyacrylamide-8 M urea gel. The amount of label incorporated at the 3' end of the RNA was compared between nonperiodate treated and periodate treated RNAs, and quantitated using a Storm Phosphorimager (Molecular Dynamics). In another measure of the amount of 3' end block that was maintained after exposure of the RNA to lysate, blocked and nonblocked RNAs were exposed to lysate under editing conditions. The RNAs were then 3'-end labeled using [α -³²P]pCp and T4 RNA ligase (Gibco-BRL), separated on a 6% polyacrylamide-8 M urea gel, and the amount of label quantitated using a Storm Phosphorimager (Molecular Dynamics). These methods indicated that approximately 94% of the *cis*-acting gRNA transcripts remained blocked after periodate treatment and subsequent incubation with a mitochondrial lysate.

Oligonucleotides

Oligonucleotides for PCR amplification, sequencing, and assay of in vitro U insertion were synthesized by standard phosphoramidite methods (Gibco-BRL), and purified from polyacrylamide-8 M urea gels. The following is a list of the oligonucleotides that were utilized in this study, and a brief description of their applications:

- S-2172: TAATACGACTCACTATAG (PCR amplification of T7 transcription templates, 5' primer);
 S-2166: TAATCATACTCCATATTTATTAAT (PCR amplification of gRNA template without oligo[U] tail, 3' primer);
 S-2167: GGGAGAATCATAATGTT (PCR amplification of gRNA template without T7 promoter, 3' primer);
 S-2010: TAATCATACTCCATATTTATTAATTAAACATTATGATTCTCCCTATAGTGAGTCGTATTA (template for PCR amplification of gRNA template with no guiding nucleotides for editing site 1);
 S-2009: TAATCATACTCCATATTTATTAATTATCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (template for PCR amplification of gRNA template with three guiding nucleotides (bold) for editing site 1);
 S-2088: TAATCATACTCCATATTTATTAATTATCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (template for PCR amplification of gRNA template with four guiding nucleotides (bold) for editing site 1);

- S-1855: TAATCATACTCCATATTTATTAATTATTTTCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (template for PCR amplification of gRNA template with seven guiding nucleotides (bold) for editing site 1);
 S-1529: TAATACGACTCACTATAGGGTTAAATTTTATTTAGCCGACTACACG (PCR amplification of mRNA template with T7 promoter, 5' primer);
 S-245: CCTCGATGTAAATACCCAATAATTAC (PCR amplification of 165 nt ND7 mRNA substrate template, 3' primer);
 S-1679: TCCGAGAGCGAAGAAACATAATGTT (PCR amplification of 65-nt ND7 mRNA substrate, 3' primer);
 S-2406: GGGTTAAATTTTATTTAGCCGACTACACG (PCR amplification of mRNA template without T7 promoter, 5' primer);
 S-2079: CCCTATAGTGAGTCGT (normalization for primer extension assay);
 S-2413: CCCTATAGTGAGTCGTATTA (blocking of normalization for primer extension assay);
 S-2919: AGCCGACTACACGATAAATAAAGTCTAACATTATGATTCTC (PCR amplification of a stabilized 3' end gRNA template with three guiding nucleotides (bold) for editing site 1, 3' primer);
 S-3020: TAATACGACTCACTATAGGGAGAATCATAATGTT (asymmetric PCR amplification of the 5' *cis* gRNA transcript for primer extension assay).

Cell culture and preparation of mitochondria

L. tarentolae (UC Strain) cells were grown to a late log phase density of approximately 1.2×10^8 cells/mL as described previously (Simpson & Braly, 1970). After washing twice with 250 mM sucrose, 20 mM Tris (pH 7.9), 2 mM EDTA (STE), the cells were resuspended at 1.2×10^9 cells/mL and swollen in a hypotonic medium of 1 mM Tris (pH 7.9), 1 mM EDTA at 4 °C. Cells were then broken by passage through a 26G needle (Becton Dickinson) at 80 psi, and after breakage the solution was brought to an isotonic concentration of 250 mM sucrose. The broken cells were then pelleted, resuspended in 250 mM sucrose, 20 mM Tris (pH 7.9), 5 mM MgCl₂ (STM), digested with DNase I (Sigma) to remove nuclear DNA, then washed by centrifugation in STE. This crude mitochondrial fraction was resuspended in 85% Percoll (Pharmacia), 250 mM sucrose, 10 mM Tris (pH 7.9), 0.1 mM EDTA. A purified mitochondrial fraction was isolated after centrifugation in a 20–35% Percoll gradient (Harris et al., 1990), washed with STE, then resuspended at 500 mg/mL in 20 mM HEPES (pH 7.5), 20 mM KCl, 10% glycerol, and lysed by the addition of Triton X-100 to a final concentration of 0.3% for use in in vitro editing reactions.

Lysate-mediated in vitro editing reactions and primer extension assay

In vitro U insertion assays were performed as described previously (Byrne et al., 1996). T7 transcribed RNAs were denatured by heating for 5 min at 65 °C in 12 mM HEPES (pH 7.5) and 0.1 mM EDTA in a 20 μ L volume, then allowed to anneal at 27 °C for 10 min. Reactions were initiated by addition of 30 μ L of a mixture containing 33.3 mM KCl, 5 mM potassium phosphate buffer (pH 7.4), 33.3 mM DTT, 1.7 mM ATP, 1.7 mM GTP, 1.7 mM UTP, 3.3 mM spermidine, and 5 μ L

of mitochondrial lysate as described above. After incubation at 27 °C for 40 min, reactions were terminated by the addition of proteinase K and SDS followed by incubation at 37 °C for 15 min, then extraction with phenol. Input RNAs were reverse transcribed, then amplified by an asymmetric PCR using the appropriate oligos as described previously, and the asymmetric PCR product purified from an 8% polyacrylamide-8 M urea gel.

Primer extension analysis of the first editing site was performed basically as described previously (Byrne et al., 1996), using S-1679 to extend into editing site 1 and S-2079 for normalization. However, instead of using a mixture of ddT-blocked S-2079 to decrease the intensity of the normalization signal, we used an overlapping blocking oligonucleotide (S-2413). This method gave a linear response of the normalization signal when the assay template was varied from 0.3 to 3.3 times the standard amount (data not shown). Primer extension assays were performed in 10- μ L reactions containing 1.5 pmol of assay template, 10 pmol of assay primer, 0.6 pmol of the normalization primer S-2079, 10 pmol of the normalization blocking oligonucleotide S-2413, 100 mM NaCl, 20 mM MgCl₂, 40 mM Tris (pH 7.5), 4 mM DTT, 0.625 μ M [α -³²P]dATP (800 μ Ci/mMol), and 1 U of Sequenase enzyme. Template DNA and oligos were first heated at 65 °C then annealed for 3 min at 50 °C with the NaCl, MgCl₂, and Tris. Primer extensions were initiated by addition of [α -³²P]dATP and enzyme, and terminated by addition of 10 μ L of formamide prior to electrophoresis in a 12% polyacrylamide-8 M urea gel.

Imaging and quantitation was performed with a Storm Phosphorimager (Molecular Dynamics). For quantitation and comparison of U insertion activity, band intensities were first normalized by analysis of the normalization signals. Specific U insertion signals were then compared by giving the most intense band (taking into account normalization) a value of 100 arbitrary units and then measuring other bands as a percentage of the most intense band.

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