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Chimeric and truncated RNAs in *Trypanosoma brucei* suggest transesterifications at non-consecutive sites during RNA editing

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ABSTRACT

RNA editing adds and removes uridines at specific sites in several mitochondrial transcripts in kinetoplastid parasites probably as specified by guide RNAs (gRNAs) that are complementary to the final edited sequence. Editing has been postulated to involve transesterification which predicts (1) chimeric molecules with a gRNA covalently attached by its non-encoded oligo U tail to an internal editing site in the mRNA and (2) the corresponding truncated 5′ portions of the mRNAs. We have characterized cDNAs representing a large number of both types of intermediates from *Trypanosoma brucei*. The lengths of both U tails and encoded gRNA sequences vary greatly in length. The majority of encoded gRNA sequences are shorter than predicted based on their minicircle coding sequences. Analysis of the predominant sites of gRNA attachment in chimeras suggests that the transesterifications that religate the truncated 5′ mRNAs may proceed more rapidly at editing sites at the 5′ end of an editing domain and at sites of U deletion. Partially edited sequences in the mRNA portion of chimeras and at the 3′ ends of truncated 5′ mRNAs also indicate a non-consecutive order of site selection during RNA editing.

INTRODUCTION

Many mitochondrial transcripts in kinetoplastid parasites undergo posttranscriptional uridine insertion and deletion at specific sites through a process called RNA editing (1–4). Editing frequently creates initiation (5–9) and termination (6–10) codons in transcripts lacking them and eliminates internal frameshifts (5,11). In addition, transcripts from several *Trypanosoma brucei* genes are so extensively edited that the protein coding sequence is essentially created by editing (6–10). The sequence information which directs RNA editing is thought to be specified by small transcripts called guide RNAs (gRNAs) which are complimentary (allowing G:U basepairs) to portions of the edited sequence (12). gRNAs are encoded on maxicircles and minicircles of kinetoplast (mitochondrial) DNA (kDNA; 6,7,12–14).

The precise mechanism by which RNA editing takes place in kinetoplastids is unknown. Two unanswered questions are 1) how are sites selected for RNA editing?, and 2) what are the chemical steps of each cycle of editing? Several models have been proposed by which gRNAs direct the progression of editing cycles along the mRNA. The 'mismatch recognition' model (12) postulates a strict 3′ to 5′ progression of editing in which the site to be edited is that immediately 5′ to the mRNA/gRNA duplex. Editing at this site then advances the duplex 5′ (on the mRNA), and the cycle is repeated at the next mismatched basepair. A second model (15) proposes that the mRNA is randomly edited within specific domains until it is complimentary to the gRNA. Subsequent gRNA/mRNA hybridization then protects this region of mRNA from being further edited. A third model, the 'dynamic interaction model' (16), predicts site selection based on regions of lower thermodynamic stability followed by progressive realignment of gRNA and mRNA as editing proceeds until the most stable structure, the gRNA/mRNA duplex, is formed. Site selection according to this model is flexible, but not random, and does not predict a strictly consecutive 3′ to 5′ progression of editing.

The chemical reactions within one cycle of editing are thought to entail cleavage, uridine addition and deletion, and religation (2,4,17). This process may involve several enzymatic activities including an endonuclease, a terminal uridylyl transferase (TUTase), a 3′ exonuclease, and an RNA ligase. Both TUTase and RNA ligase activities have been detected in kinetoplastid mitochondrial lysates (18,19). Models proposing separate enzymes for each of these steps suggest that Us added to mRNA during editing are derived from UTP. An alternative proposal is that editing is performed by a single active site via a transesterification mechanism (20,21) similar to that involved in some types of RNA splicing (22). In this model, the non-encoded 3′ oligo U tail of the gRNA attacks the site to be edited and is itself the source of Us. The predicted intermediates in the editing process differ depending on which mechanism is invoked. If editing occurs by transesterification, chimeric molecules with a gRNA covalently attached to the 3′ portion of an mRNA and the corresponding truncated 5′ portions of the mRNAs should

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be detectable in steady state RNA populations. Such gRNA-mRNA chimeras have been detected in both *Leishmania tarentolae* (21) and in *T. brucei* (16) where editing is more extensive, and truncated 5′ cytochrome oxidase subunit III mRNAs have been detected in *T. brucei* (15). We report here the sequences of several gRNA-mRNA chimeras for two regions of the *T. brucei* ATPase subunit 6 (A6) transcript (6). We also report the characteristics of truncated 5′ RNA molecules from both A6 and CR4 (23) transcripts. The characteristics of some of these molecules suggest that the order of site selection does not proceed strictly 3′ to 5′. These molecules appear to be editing intermediates, and their existence is consistent with the transesterification mechanism of RNA editing.

MATERIALS AND METHODS

Cell culture, mitochondrial isolation and RNA extraction

T. brucei brucei clone IsTar 1 from stock EATRO 164 was grown as previously described (24). Mitochondria were isolated from $0.5-2.0\times10^{11}$ cells by the method of Harris *et al.* (19) and stored at −70°C until RNA extraction. RNA was isolated from either whole cells or mitochondria by the acid guanidinium-phenol-chloroform method (25).

Oligonucleotide primers

The following oligonucleotides were used in this study; restriction sites incorporated at their 5′ ends are underlined.

gA6-149c	CAGGAATTCACAACAAGAGACGAATAGAAAAG
gA6-14c	CAGGAATTCCGATAACGAATCAGATTTTGAC
A6-17	AGTGGATCCGCAAAAACGTCGACAAACAACG
MURF 4-7	GCGGATCCGATCTTATTCTATAACTCC
A6-5′	AAGCTTAAGCTTAAAAATAAGTATTTTGATATTATTAAAG
CR4U-5′	CGGAATTCATAATTTATTGTTATCTTTGTG
CR4U-3′	CGCCTAGGAAACAAAACTATTTCTTCGC
B-dG_{10}	CGGGATCCGGGGGGGGGG
KS	CGAGGTCGACGGTATCG
ZR	CGGTGGCGGCCGCTCTA

PCR amplification of gRNA-mRNA chimeras

Protocol I: 2.5 μg of procyclic form (PF) mitochondrial RNA was annealed with 500 ng of A6-17 for 3 mins. at 70°C, 10 mins. at 37°C, and 10 mins. at 25°C. cDNA was synthesized at 37°C using MMLV reverse transcriptase (Superscript; BRL) according to manufacturer's instructions. 25% of the cDNA product was then amplified by PCR with A6-17 and gA6-149c for 30 cycles of 94°C denaturation, 45°C annealing, and 72°C extension. Five μl of the resulting PCR product was then re-amplified under the same conditions, and cloned as described below.

Protocol II: cDNA was synthesized from 3 μg PF mitochondrial RNA using the A6-17 primer for 15 mins. at 70°C using *rTth* thermostable reverse transcriptase (Perkin-Elmer Cetus) in the presence of 1 mM $MnCl_2$. The $MnCl_2$ was chelated and $MgCl_2$ added to 2 mM, and the entire cDNA synthesis was then PCR amplified using A6-17 and gA6-149c for 35 cycles of 94°C denaturation, 60°C annealing, and 72°C extension.

Protocol III: Five μg of PF mitochondrial RNA was annealed with 500 ng MURF 4-7 for 3 mins. at 95°C, 10 mins. at 37°C, and 10 mins at 25°C. The entire cDNA reaction was then PCR amplified using MURF 4-7 and gA6-14c as described in Protocol I.

PCR amplification of truncated 5′ molecules

To obtain cDNAs from 5′ truncated A6 mRNA, 10 μg total bloodstream form (BF) RNA was C tailed using poly A polymerase as previously described (26), hybridized with 100 ng B-dG_{10}, and first strand cDNA synthesized using MMLV reverse transcriptase at 37°C. 15% of the product was then PCR amplified with B-dG_{10} and A6-5′ with 30 cycles of denaturation at 94°C, annealing at either 45°C (first four cycles) or 50°C (following 26 cycles), and extension at 72°C.

To obtain cDNAs from 5′ truncated CR4 RNA, 10 μg total PF RNA was C tailed, and first strand cDNA was synthesized as described above using B-dG_{10}. 25% of the cDNA was then PCR amplified using B-dG_{10} and CR4U-5′ for 25 cycles of 94°C denaturation, 45°C annealing and 72°C extension.

Cloning and sequence analysis

PCR products were digested with appropriate restriction enzymes, size selected on a 1.5% agarose gel (except CR4 5′ truncated molecules), transferred to NA45 paper (S&S), and ligated into pBluescript II SK− (Stratagene). Ligation mixture containing CR4 5′ truncated molecules was used to transform *E. coli* SURE cells (BRL); all other ligation mixtures were used to transform *E. coli* DH5α F′IQ competent cells (Life Technologies, Inc.). Recombinants containing 5′ truncated CR4 molecules were identified by colony hybridization with a probe generated by PCR amplification of a plasmid containing the CR4 gene (pTKHR38; 27) with CR4U-5′ and CR4U-3′ in the presence of α[^{32}P]-dATP (9). All other recombinants were identified by colony PCR with the vector-specific primers KS and ZR. Recombinant plasmids were sequenced using the dideoxy chain termination method using Sequenase (USB) according to the manufacturer's instructions and analysis on polyacrylamide gels or by automated sequencing on an Applied Biosystems Model 373A DNA sequencer. The resulting sequences were analyzed using DNASTAR (Madison, WI) and ESEE (E. Cabot, Univ. of Rochester).

RESULTS

gRNA-mRNA chimeras

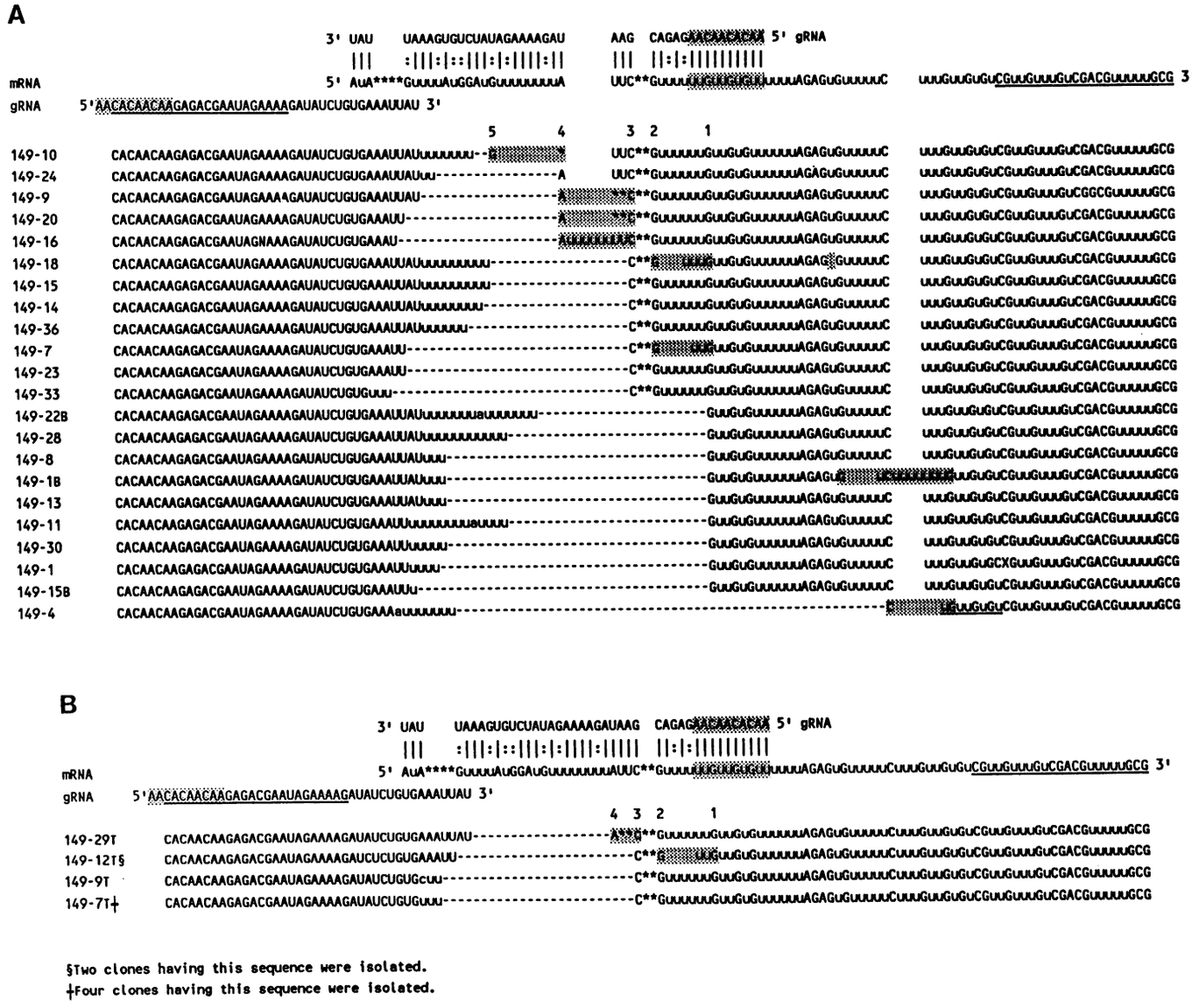
Numerous gRNA-mRNA chimeras were obtained for two regions of A6 mRNA (Figs. 1 and 2). These are the furthest 3′ edited region (Fig. 2) and a region where Us 3′ to a C are deleted (Fig. 1). The chimeras were obtained by PCR amplification using 5′ primers for gA6-14 or gA6-149 gRNAs and 3′ primers for edited A6 mRNA sequence just downstream of the regions specified by these gRNAs. The gA6-14 and gA6-149 gRNAs are encoded in minicircles and were identified by computer analysis (16). Their existence in kinetoplast mRNA (kRNA) was shown by Northern blotting (28). The furthest 3′ editing sites (ESs; positions between non-uridine nucleotides) to which they can duplex are ES14 and 149, respectively (see 28 for nomenclature). These regions were selected to explore the initial edited region and the complex situation where Us are deleted 3′ to Cs (4).

The gRNA portion of the chimeras varies substantially in length. Of 30 chimeras sequenced in the gA6-149 region, only 13 contain the entire minicircle sequence that is complimentary to edited A6 mRNA (Fig. 1). The rest lack 2−7 nucleotides (nts) of encoded sequence in the 3′ region of the gRNA. This incompleteness of gRNAs is even more striking in chimeras from the gA6-14 region (Fig. 2). No chimeras in this region were

obtained with a gRNA of the expected length based on the number of nts in the minicircle sequence which could duplex with edited A6 mRNA. gRNAs from chimeras in this region are between 1 and 15 nts shorter than the length predicted from the minicircle sequence.

The length of the U tracts that join the gRNA and mRNA sequences in the chimeric molecules is also highly variable. The U tract length ranges from 1 to 15, and averages 5, including 3′-most Us which could be encoded in the gRNA. In addition, three chimeras, 149-11, 149-4, and 149-22B, have U tracts containing one A residue. While the A is at the 5′ end of the U tract in 149-4, As are found near the middle of the two longest U tracts in 149-11 and 149-22B. Perhaps the presence of these As reflects non-specific incorporation of A by TUTase or occasional A addition to gRNAs by poly A polymerase. There does not appear to be a strict correlation between U tract length and the size of the encoded gRNA sequence or the site of gRNA attachment (see below). However, longer gRNAs tend to have longer non-encoded U tracts (Fig. 1 and 2).

The gRNAs are attached at ESs in the 3′ region of the editing domain defined by the gRNA in all chimeras examined. The A6-14 region has 16 possible ESs. (ESs in Figs. 1 and 2 are



§Two clones having this sequence were isolated.
†Four clones having this sequence were isolated.

Figure 1. Nucleotide sequences of gRNA-mRNA chimeras from the gA6149 region predicted by cDNAs were generated by cDNA synthesis at 37°C and PCR amplification protocol I **(A)** or by cDNA synthesis at 70°C and PCR amplification protocol II **(B)**. The region of fully edited A6 mRNA that is thought to be specified by gA6149 plus sequence extending 3′ to the A6-17 primer is shown. ESs are numbered beginning with the presumed 3′-most ES specified by gA6149. The sequence of gA6149 is shown above the gRNA portion of the chimeras in the 5′ to 3′ direction and also above the mRNA in the 3′ to 5′ direction with the anchor duplex shaded to illustrate gRNA-mRNA complementarity . Oligonucleotide primer locations are underlined. The shaded regions in the cDNA clones represent partially edited sequence (edited sequence not matching fully edited). The 8 nt underlined in 149-4 indicate the putative anchor used in generating this chimera (see text). Lower case indicates Us and As not encoded in either the gRNA or mRNA gene. Asterisks, encoded Us deleted by editing; |, Watson–Crick basepairing; :, G-U basepairing. Dashes are inserted for purposes of alignment. The sequences of clones 149-18 and 149-7 have been previously reported (16).

numbered beginning with the 3′-most site whose editing is thought to be specified by the respective gRNA). The gRNA is attached at ES 1 in 25% of chimeras sequenced for this region while it is attached at ES 2 in the remaining chimeras (Fig. 2). In chimeras for the gA6-149 region generated at 37°C (Fig. 1A), 41% of the gRNAs are attached at ES 1, 32% at ES 3, 18% at ES 4, and 4.5% (1 chimera) at ES 5. No chimeras were obtained with gRNAs joined at ESs 2 or 6-11. One chimera, 149-4, has the gRNA attached five ESs 3′ to the usual 10 nt anchor region for gA6149. This molecule probably arose by anchoring of gA6149 at a region just 3′ of the gRNA attachment site to which it could form an 8 nt anchor (underlined in Fig. 1A) with fully edited A6 mRNA. In addition, clone 149-10 (Fig. 1A) has a non-U nt deletion which may represent a PCR or cloning artifact.

The possibility that the skewed distribution of gRNA attachment may reflect the greater stability of larger duplexed regions between the gRNA and more fully edited mRNA and the consequent inability of reverse transcriptase to synthesize cDNA from these regions (21) was investigated. cDNA was synthesized from kRNA at 70°C using *rTth* thermostable reverse transcriptase and the A6-17 primer, and PCR amplified with A6-17 and gA6149c (Fig. 1B). Performing the reverse transcriptase reaction at 70°C should prevent hybridization of the gRNA with complimentary sequence in the mRNA, thereby allowing cDNA synthesis even in chimeras with long potential gRNA-mRNA duplex regions. However, this protocol failed to generate any chimeric clones with gRNAs attached at more 5′ sites (Fig. 1B). Chimeras obtained using the thermal reverse transcriptase protocol differ from those generated at 37°C only in the absence of gRNAs attached at ES 1. Of 8 chimeras generated by cDNA synthesis at 70°C, 7 have gRNAs attached at ES 3 and one has the gRNA attached at ES 4. Therefore, the preponderance of chimeras with gRNAs attached at more 3′ ESs does not appear to be due to the stability of the duplex formed between gRNA and more extensively edited mRNA, and thus may reflect the actual kinetics of the editing process.

In chimeras in which gRNAs are attached at ESs other than ES 1, editing of the mRNAs is not always as expected for fully edited A6. Of 30 chimeras joined at such positions, 11 contain partially edited mRNA sequence (edited sequence not matching fully edited; 16), shown as shaded regions in Fig. 1. This includes A6149-4 in which the mRNA sequence does not match that expected if the mRNA were fully edited by gA6149 which had anchored at the 8 nt anchor. In addition, two chimeras, 149-18 and 149-1B, contain partially edited mRNA sequence 3′ to the anchor duplex region.

Truncated 5′ mRNAs

A truncated molecule comprising the portion of the mRNA 5′ to the gRNA attachment site is the predicted counterpart of a chimera if editing occurs via transesterification. In order to detect such molecules, total RNA was C tailed, cDNA synthesis was primed with B-dG_{10}, and the resulting cDNAs were amplified with B-dG_{10} and a primer corresponding to unedited 5′ sequence of either A6 or CR4 mRNA. We could not definitively establish whether cDNAs generated in this manner represent editing intermediates or simply molecules broken during RNA isolation. However, we can predict certain characteristics of truncated 5′ editing intermediates. Molecules containing exclusively unedited sequence would be a type of putative intermediate due to the general 3′ to 5′ progression of editing (29). In addition, molecules having at their 3′ ends a junction region containing partially edited ESs interspersed with fully edited ESs would also be expected editing intermediates, since junction regions are believed to be regions of active editing (8,16).

Figure 3 shows a schematic representation of truncated 5′ A6 and CR4 mRNAs. Of the A6 clones, five contain only unedited ESs (Fig. 3A). However, one of these (A6-32) has two Us at its 3′ end which are not encoded by the A6 gene, suggesting that the RNA was cleaved within a previously edited site. Three of the 14 A6 5′ truncated clones display partially edited sequence no more than 3 ESs from their 3′ ends as predicted for an RNA cleaved within a junction region (16). Four others contain largely fully edited sequence; however, three of these clones contain a partially edited region at their extreme 5′ ends at ESs 29, 41, and 52 ES from their 3′ ends. While this pattern suggests that these molecules were undergoing editing only at their 5′ ends at the time of RNA isolation, it is conceivable that they were being re-edited at the site of cleavage (16). We have previously described an ND7 cDNA clone with partially edited ESs separated

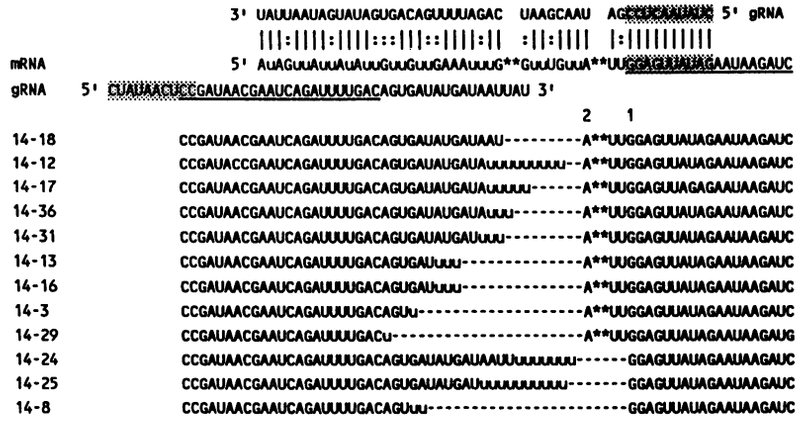


Figure 2. Nucleotide sequences of gRNA-mRNA chimeras from the gA614 region determined from cDNAs. Clones were generated by PCR amplification protocol III (Materials and Methods). Alignments and notations as in Figure 1.

by 25 fully edited ESs (16; clone 5M13), and cDNA clones with partially edited ESs separated by 80–100 fully edited ESs were frequently isolated for the ND8 gene (8). Two of the A6 clones contain anomalous sequence at their 3′ ends. One clone ends in 33 A residues followed by the sequence, CAAT. A second contains a 41 nt sequence at its 3′ end which was not identified computer in searches of *T. brucei* maxicircle, minicircle, fully edited mRNA, or vector sequence. In addition, this sequence cannot serve as a gRNA to direct editing of any known *T. brucei* edited mRNAs. Both of these clones have long stretches of completely unedited A6 sequence 5′ of the anomalous sequence.

Only a portion of the consensus edited sequence of CR4 mRNA is known (R.A.C. and K.S. unpublished). Thus, Figure 3B depicts only the regions of truncated 5′ mRNAs which overlap known consensus edited sequence. Of these, 100% contain partially edited sequence 0–5 ESs from their 3′ ends, suggesting that all of the CR4 truncated 5′ molecules arose through cleavage in a junction region.

Several truncated 5′ molecules have Us (Ts in the cDNA) at their 3′ ends. Seven A6 clones and three CR4 clones end in 1–5 Us. These clones may represent RNAs which were cleaved within a site having previously been edited. The number of Us at the 3′ end of most of these clones is equal to or less than that predicted by the fully edited sequence; however, two of the CR4 clones (CR4-87 and -78) contain more Us than would be present in the fully edited mRNA at that ES. Although we frequently observe ESs containing a greater number of Us than in fully edited mRNA (16), these sites rarely contain as many as 28 Us. This raises the possibility that truncated 5′ mRNAs may have Us added to their 3′ ends by the TUTase activity which has been reported in *T. brucei* mitochondria (19).

DISCUSSION

The results presented here are consistent with a model for RNA editing in kinetoplastids that entails transesterification. Both gRNA-mRNA chimeras and the corresponding truncated 5′ portions of the mRNAs which are predicted products of the transesterification reaction have been detected in *T. brucei*. These molecules are potential RNA editing intermediates. A second transesterification where the 3′ hydroxyl of the truncated 5′ mRNA attacks the gRNA-mRNA chimera would religate the mRNA completing one cycle of editing. Importantly, the site of this second attack would determine the number of Us added or removed. Observance of partially edited sequences both in gRNA-mRNA chimeras 3′ to the gRNA-mRNA junction and in the 3′ region of the truncated 5′ mRNAs provides additional evidence (see 16) that editing does not proceed in a strictly 3′ to 5′ direction along the mRNA.

The significance of chimeric gRNAs which are shorter than predicted based on the length of minicircle coding sequence homologous to edited RNA is uncertain. This class of molecules was also detected in *L. tarentolae* (21), although not in as large a proportion as in the present study. Shortened chimeric gRNAs may be the result of premature termination of gRNA transcription (21), posttranscriptional events not related to RNA editing (*e.g.* nuclease action) or, intriguingly, may be a result of editing. The presence of short non-chimeric gRNAs in *L. tarentolae* (30) supports creation of these molecules through premature transcription termination or nuclease activity. However, in *L. tarentolae*, short non-chimeric gRNAs appeared to lack non-encoded oligo U tails (30) while in this organism non-encoded U tracts are present between gRNA and mRNA sequences in

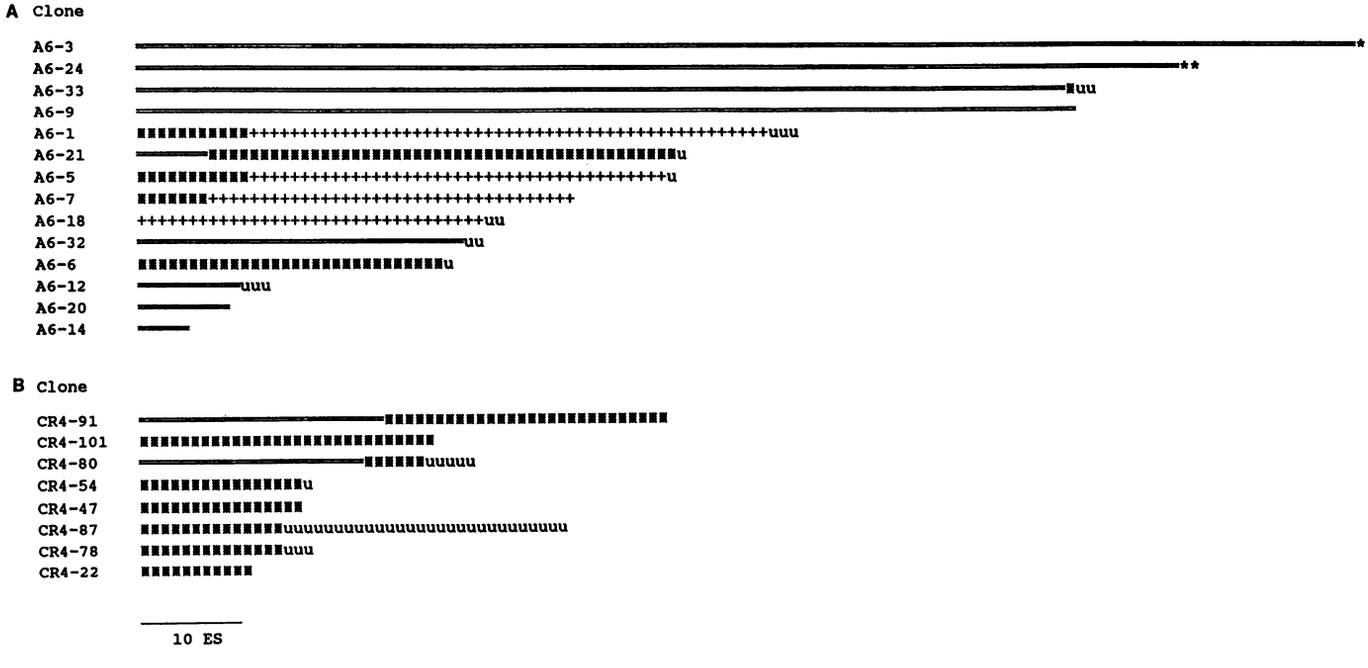


Figure 3. Schematic representation of A6 **(A)** and CR4 **(B)** truncated 5′ mRNAs as determined from cDNA sequences. RNAs are shown in a 5′ to 3′ direction. A6 sequences begin with the first nt following the 5′ PCR primer and end at the site of the C tail. CR4 sequences begin with the 5′ most nt of the consensus sequence known to date and end with the site of the C tail. =, unedited region; ■ ■, partially edited region; + +, fully edited region; u, 3′ uridines; *, 41 nt anomalous sequence (see text); **, polyadenosine anomalous sequence (see text). The bar at the bottom indicates 10 potential ES.

short gRNA-containing chimeras (21). The short gRNA-containing chimeras described here all also have such U tracts, and in the majority of cases these Us could not have been encoded by either the gRNA or mRNA gene. If post-transcriptional U addition requires a properly terminated gRNA (30), this suggests that gRNAs are full length when they first transesterify and become shortened during the editing process. However, at present there is not enough evidence to allow us to distinguish between the various mechanisms by which short chimeric gRNAs could arise. It is possible that the abundance of chimeras containing short gRNAs in the steady state RNA population does not reflect their ability to participate in further steps of the editing process. That is, they may be 'dead end' molecules which are unable to undergo the second reaction involving invasion of the truncated 5′ mRNA. Providing that these chimeras can participate in the second reaction, short gRNAs could easily be functional since they could edit the 3′ portion of an editing domain, the 5′ end of which could subsequently be edited by a full length gRNA. In addition, overlapping gRNAs that could perform a proofreading function have been identified for some domains of several edited mRNAs (28).

The U tracts observed in chimeras from *T. brucei* (ranging from 1−15 and averaging 5 nt) are shorter than those in chimeras from *L. tarentolae*, which range from 1−27 and average approximately 12 nt (21). It is interesting in this context that the overall number of Us added by RNA editing in *T. brucei* is much greater than in *L. tarentolae* (4). Thus, the total mass of gRNAs may be significantly higher in *T. brucei*. Assuming a cell has a finite capacity to produce the TUTase activity which is believed to add 3′ Us to gRNAs, shorter U tracts may reflect the relative difficulty of TUTase in keeping pace with the transfer of Us from gRNA to mRNA in *T. brucei vs. L. tarentolae*.

Examination of chimeras provides evidence regarding the kinetics of editing. Chimeras isolated from the steady state RNA population are biased toward those with the gRNA attached to a site near the 3′ end of the respective editing domain. While this bias may be an artifact stemming from the difficulty of synthesizing cDNA from a region of gRNA-mRNA duplex (21), this does not appear to be the case since a similar bias is observed even when cDNA is synthesized at 70°C. We have previously reported generation of a gRNA-mRNA chimera having a 35 nt duplex region by cDNA synthesis at 37°C. (16; clone 5M14). Even allowing for differences in base composition, this suggests that an increase in temperature to 70°C would allow cDNA synthesis from the full repertoire of gRNA-mRNA chimeras. Assuming that the 3′ bias of gRNA attachment reflects the actual kinetics of editing, our results suggest that the second transesterification with the corresponding truncated 5′ mRNA proceeds more rapidly during editing at the 5′ end of an editing domain than at the 3′ end. The secondary structures of the chimeras may be responsible for this phenomenon. A gRNA attached near the 5′ end of a nearly fully edited domain is predicted to form a substantial stable duplex with mRNA, leaving only a small region of RNA that is single-stranded (16). In contrast, chimeras with gRNAs attached at the 3′ end of the editing domain are not predicted to form substantial gRNA-mRNA duplexes (outside the 'anchor' region), but are predicted by the Zucker FOLD program (31) to have scattered short duplexes and hence substantial single-stranded RNA (data not shown). Thus, chimera secondary structure may affect the re-invasion rate of the truncated 5′ mRNA. For example, if by extension of our previous editing model (16) re-invasion of the truncated 5′ mRNA takes place at a region of low thermodynamic stability (*i.e.*, single-strandedness), then a chimera with the gRNA attached at a 5′ site might have a more constrained catalytic center. Additional components of the editing machinery may also affect the kinetics of the first (gRNA) and second (truncated 5′ mRNA) transesterifications.

The absence of chimeras with gRNAs attached at ES 2 in the gA6149 region, which is a site of U deletions, is also striking (Fig. 1). Re-invasion of the truncated 5′ mRNA may occur very rapidly at ES2 since chimeras with gRNA at this site are very low in steady state kRNA populations. Deletion of Us by a transesterification mechanism requires that the truncated 5′ mRNA attack the chimera 3′ to a non-basepaired U rather than 5′ to a basepaired U as is the case for U additions (20). The U deletion reactions may be inherently more rapid than the U additions. Another possibility is that the Us may be deleted from this site by the previous (*i.e.*, more 3′) gRNA (see 16), and thus no chimeras with gA6149 attached at this site were detected.

ESs where gRNAs are attached to mRNAs are presumably sites undergoing editing at the time of RNA isolation. Thus, chimeras provide insight into the order of editing events. Analysis of partially edited mRNAs from *T. brucei* strongly suggests that editing does not proceed in a strictly consecutive 3′ to 5′ direction (16), and this conclusion is supported by analysis of gRNA-mRNA chimeras and truncated 5′ mRNAs. Approximately one-third of the chimeras with gRNAs joined at positions other than ES 1 are partially edited at sites within the editing domain of the attached gRNA. Thus, if meaningful mRNA is to be ultimately produced from these chimeras, editing must occur 3′ to the site of gRNA attachment. In addition, partially edited sites must be re-edited (16). Isolation of truncated 5′ mRNAs with partially edited sequence at their 3′ ends (Fig. 3) also suggests that a given editing event can occur 3′ to a previous editing event.

Interestingly, two chimeric clones were isolated which contain partially edited sequence 3′ to the anchor region for the gRNA component of the chimera (Fig. 1A). The mRNA components of these chimeras may be re-edited by the gRNA specifying the edited sequence 3′ to that specified by gA6149. This suggests that a gRNA can direct editing of a region before the previous (*i.e.*, more 3′) gRNA has completed direction of editing of its corresponding region of mRNA. Thus, at least two gRNAs can apparently direct editing simultaneously, as we had previously predicted based on the presence of junction regions spanning up to 105 nt (16).

The characteristics of both chimeras and truncated 5′ mRNAs suggest mechanisms by which partially edited mRNAs can arise. Transesterification of a gRNA containing fewer 3′ Us than necessary for complete editing of an ES would generate an ES having less than the fully edited number of Us (*e.g.*, shaded ESs 3′ of the gRNA attachment site in clones 149-18, 149-7, and 149-12T in Fig. 1). This type of transesterification has presumably occurred in chimeras 149-24, -9, -20, -16, -8, -1B, -13, and -15B (Fig. 1A) in which subsequent invasion of the truncated 5′ mRNA would result in ESs with fewer than the fully edited number of Us. Furthermore, if these gRNAs are to remain functional, this implies that Us are continuously added to the 3′ end of gRNAs as editing of a given domain progresses. An ES with greater than the fully edited number of Us could arise through transesterification of a gRNA with more Us than required for complete editing of the site. This would depend on the interaction of the U tract with the gRNA and the mechanism determining where the truncated 5′ mRNA will invade. Another

interesting possibility is that 3′ Us could (also) be added to the truncated 5′ mRNAs by TUTase, as suggested by the presence of a CR4 truncated 5′ molecule containing 28 Us at its 3′ end. This mechanism for generating ESs with greater than the fully edited number of Us is attractive since it is independent of any gRNA-mRNA hybridization that may guide the number of Us added.

While the presence of gRNA-mRNA chimeras does not constitute proof of a transesterification mechanism of RNA editing, it is highly suggestive. Verification of this model will require development of an *in vitro* editing system, allowing us to test whether Us are transferred from the 3′ end of gRNAs to edited mRNAs. Such a system will also allow us to identify factors other than mRNA and U-tailed gRNA, if any, which are required for transesterification.

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