MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 2916–2924 Vol. 15, No. 6
0270-7306/95/$04.00+0

Editing Domains of *Trypanosoma brucei* Mitochondrial RNAs Identified by Secondary Structure

KENNETH J. PILLER,[1] CAROLYN J. DECKER,[1,2]† LAURA N. RUSCHÉ,[1] MICHAEL E. HARRIS,[3]‡ STEPHEN L. HAJDUK,[3] AND BARBARA SOLLNER-WEBB[1,2]*

Department of Biological Chemistry[1] and Human Genetics Program,[2] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and Department of Biochemistry, School of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294[3]

Received 13 December 1994/Returned for modification 8 February 1995/Accepted 17 February 1995

The posttranscriptional insertion and deletion of U residues in trypanosome mitochondrial transcripts called RNA editing initiates at the 3′ end of precisely defined editing domains that can be identified independently of the cognate guide RNA. The regions where editing initiates in *Trypanosoma brucei* cytochrome *b* and cytochrome oxidase subunit II preedited mRNAs are specifically cleaved by a trypanosome mitochondrial endonuclease that acts like mung bean nuclease and therefore is single strand specific. The regions where editing initiates in virtually all examined preedited mRNAs are predicted to form loop structures, suggesting that editing domains could generally be recognized as prominent single-stranded loops. In contrast to preedited mRNA, edited mRNA can be either resistant or sensitive to cleavage by trypanosome mitochondrial endonuclease, depending on the reaction conditions. This selectivity appears dependent on the availability of extract RNAs, and in model reactions, edited mRNA becomes resistant to cleavage upon base pairing with its guide RNA. Natural partially edited mRNAs are also specifically cleaved with a sensitivity like preedited and unlike edited mRNAs, consistent with their being intermediates in editing. These results suggest that in vivo, the structure of editing domains could initially be recognized by the mitochondrial endonuclease, which could target its associated RNA ligase and terminal U transferase to begin cycles of enzymatic editing modifications.

Many mitochondrial transcripts in trypanosomes and other kinetoplastids are specifically modified by the insertion and less frequent deletion of U residues, in a posttranscriptional process termed RNA editing (15, 17, 34, 39). Editing is required for the formation of functional mRNAs by creating protein-coding sequence, correcting encoded frameshifts, supplying potential translation start and stop signals, and/or extending open reading frames. The editing modifications occur within editing domains that vary in length from a few nucleotides to the entire coding region, and editing can account for over half the residues of the mature mRNA. The precise introduction of these U alterations occurs by a still unknown mechanism and appears to be without precedent.

Small mitochondrial guide RNAs (gRNAs) bearing complementarity to segments of edited sequence (allowing for G · U base pairing) evidently provide the information for the U insertions and deletions (4). The appropriate gRNA presumably binds to the editing substrate (the preedited mRNA) by base pairing between its 5′ portion and the region of the preedited mRNA just 3′ of the editing domain (Fig. 1). The U insertions and deletions in the mRNA would then be directed by the creation of additional base pairing in the editing domain (4). This process would cause editing to progress in a 3′-to-5′ direction, as is observed. Once the editing is completed, the gRNAs may remain duplexed with the mRNA.

* Corresponding author. Mailing address: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-7419. Fax: (410) 955-0192. Electronic mail address: Barbara.Sollner-Webb@qmail.bs.jhu.edu.

† Present address: Department of Biology, Indiana University, Bloomington, IN 47405.

‡ Present address: Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721.

The finding that the 3′ oligo(U) tail of the gRNA can become covalently joined to the downstream portion of the mRNA at an editing site suggested that these gRNA-mRNA chimeric molecules were editing intermediates and that the added U residues derive from the 3′ end of the gRNA (5, 6). The editing mechanism was thus proposed to involve sets of transesterification reactions (6, 7) (Fig. 1), akin to intron removal from mRNA. However, sets of endonuclease cleavage and RNA ligation reactions (Fig. 1), akin to intron removal from tRNA (23), could produce the same gRNA-mRNA chimeras, and partially edited RNAs and therefore could alternatively be the mechanism of RNA editing (16, 34). Notably, *Trypanosoma brucei* mitochondria contain an endonuclease activity that is specific for preedited mRNAs, targeting preedited but not edited cytochrome *b* (CYb), cytochrome oxidase subunit II (COII), and COIII transcripts and cleaving in the region where editing initiates (16); *Leishmania tarentolae* mitochondria may contain a similar endonuclease (32). Trypanosomatid mitochondria are also unusual in that they contain abundant RNA ligase activity (1, 25), which has not been observed in mitochondria of other organisms (7a, 22a, 44). Thus, the activities postulated to catalyze editing in the enzymatic scheme are present in trypanosomatid mitochondria. The recent demonstration that the gRNA-mRNA chimera formation observed in *T. brucei* extracts is catalyzed by the mitochondrial endonuclease and RNA ligase (24, 29) is consistent with the enzymatic editing mechanism.

Although gRNAs are thought to direct the actual editing reaction, analysis of partially edited mRNAs indicates that the editing domains can initially become identified independently of the gRNA. In the vast majority of partially edited RNAs present in *T. brucei* (references 11, 20, 21, 28 and references therein) and numerous partially edited RNAs in *L. tarentolae* (41), the junction between the correctly edited 3′ region and the not yet edited 5′ region is complex, containing incorrect

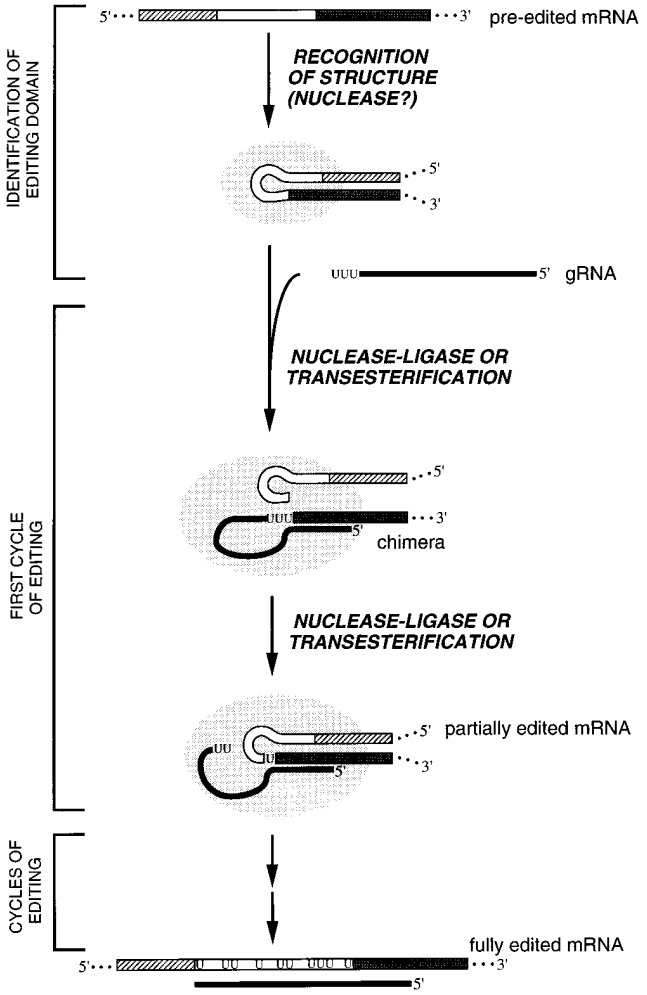

FIG. 1. Trypanosome RNA editing. The upper portion represents identification of the preedited RNA region where editing will begin, shown in this study to be likely due to recognition of the secondary structure of the mRNA by extract components (grey oval), possibly the mitochondrial endonuclease. Below are illustrated models for cycles of editing involving formation of gRNA-mRNA chimeras and re-formation of partially edited mRNA, proposed to occur either by transesterification (6, 7) or by endonuclease and RNA ligase reactions (34).

numbers of U residues at multiple sites, including ones that are not edited in the mature RNA. In these molecules, the aberrant editing can begin precisely at the 3′ end of the editing domain, yet aberrant editing is strikingly absent from the domains that do not become edited in the mature RNA, indicating that aberrant and faithful editing are both directed to begin at the same position in the mRNA (11). Most aberrant editing evidently arises from faithfully editing but using an incorrect gRNA, and in some cases the incorrect gRNA has been identified as a correct gRNA for another mRNA (9, 40, 42). The use of an incorrect gRNA has been shown for numerous examples, including the 3′ aberrant editing discussed in reference 11, because (i) multiple independent cDNAs contain the same extensive aberrantly edited sequence, indicating that this editing was directed by something, yet (ii) misalignment of the correct gRNA could not have directed this sequence, and hence it was directed by a different guiding RNA (11). Such a specific targeting of incorrect gRNAs to the normal editing domain of the mRNA, beginning at its 3′ end, implies that the region of a mRNA where editing initiates can be identified independently of its cognate gRNA.

If not the cognate gRNA, then what features of the mRNA allow the identification of its editing domain? One possibility is that the trypanosome mitochondrial endonuclease, which shows both the substrate and site specificity to recognize editing domains in preedited mRNAs (16, 24, 29), could mimic the in vivo recognition of editing domains.

In this investigation, we studied the action of trypanosome mitochondrial endonuclease that specifically cleaves the editing domain of preedited mRNA. We demonstrate that its cleavage specificity on preedited CYb and COII mRNAs is mimicked by a heterologous single-strand-specific endonuclease, mung bean nuclease. Therefore, the mitochondrial nuclease is structure specific, cleaving in prominent single-stranded loops. The editing domains of virtually all examined preedited mRNAs can be folded to form analogous loop structures, suggesting that secondary structure may generally be a primary determinant in the initial recognition of editing domains in vivo. We also show that the selectivity of the trypanosome mitochondrial extract for cleaving preedited but not edited mRNA depends on the reaction conditions. The selectivity is observed at a high dithiothreitol (DTT) concentration and appears dependent on RNAs of the extract. Specificity for preedited over edited mRNA can also be restored to reactions at a low DTT concentration by duplexing with complementary gRNA, suggesting that gRNAs could help protect correctly edited regions from further modification in vivo. We further show that natural, aberrantly edited mRNAs are cleaved with a specificity like that of preedited mRNAs, consistent with their being substrates for further editing. We suggest that initiation of editing involves recognition of the editing domain in the preedited mRNA through its secondary structure, possibly by the single-strand-specific mitochondrial endonuclease, and this could begin cycles of endonuclease/ligase-based editing.

MATERIALS AND METHODS

CYb and COII preedited, partially edited, and edited substrate RNAs. *T. brucei* preedited and partially edited CYb cDNAs containing 20 CYb nucleotides (nt) upstream and 100 nt downstream of the editing domain were described previously (11), as was the analogous fully edited CYb cDNA (16). They are cloned between the *Mlu*I and *Pst*I sites of pIBI31 (International Biotechnologies Inc.). COII preedited and edited sequences extending 212 nt upstream and 99 nt downstream of the editing domain (stopping before the region of the putative gRNA) cloned in pBluescript KS+ (Stratagene) were also described elsewhere (16). Otherwise identical COII plasmids except containing 142 nt downstream of the editing domain, including the putative gRNA, were prepared in the same manner but using a more distal 3′ primer (5′ TTATAGATTATATACCTTTC 3′) for cDNA synthesis and PCR.

Substrate RNAs were transcribed from plasmid templates digested with *Hin*dIII, using T3 RNA polymerase (Boehringer Mannheim) and [^{3}H]CTP for quantitation. They were 5′ end labeled by treatment with calf intestinal alkaline phosphatase (Boehringer Mannheim) followed by T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP. Where noted, the RNAs were instead 3′ labeled with RNA ligase and [^{32}P]pCp. Labeled RNAs were gel purified from 4% polyacrylamide–7 M urea gels by overnight extraction at 37°C as described previously (8), extracted with phenol-chloroform (1:1), and precipitated with ethanol.

Preparation of mitochondrial extracts and in vitro mitochondrial endonuclease reactions. Mitochondria were isolated from exponentially growing procyclic trypanosomes as described previously (17). Mitochondria from 10^{10} cells (~10 mg of protein) were suspended in 1 ml of 25 mM Tris-Cl (pH 7.9)–10 mM $MgCl_2$–1 mM EDTA–60 mM KCl–0.5 mM DTT–10% glycerol–1 mM ATP. The samples were made 0.5% Triton X-100 (added from a 10% solution prepared in sterile H_2O), gently mixed, allowed to sit on ice for 5 min, and centrifuged at 10,000 × *g* in a microcentrifuge for 5 min at 4°C. The supernatant was stored in aliquots at −70°C.

Endonuclease cleavage reaction mixtures contained ~50 fmol of substrate RNA (~5×10^3 cpm of 5′-end-labeled RNA), 25 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$ or 10 mM magnesium acetate, 1 mM EDTA, and 2.5 μl of extract in a total volume of 22 μl. For the relaxed conditions, the reaction mixtures also contained 100 mM KCl and 50 μg of heparin (Sigma) per ml. For the stringent

conditions, the reaction mixtures also contained 10 mM DTT, 10% glycerol, and 60 mM KCl. (Inclusion of heparin augments cleavage of both edited and preedited mRNA in the absence of DTT but markedly suppresses cleavage in the presence of DTT.) Following incubation at 25°C for 30 to 60 min, reaction mixtures were brought to 100 μl and 0.3 M sodium acetate, supplemented with 1 to 5 μg of yeast tRNA, extracted with phenol-chloroform (1:1) and chloroform, and ethanol precipitated. They were suspended in deionized formamide containing 20 mM EDTA and 0.05% xylene cyanol-bromphenol blue, denatured at 95°C for 3 min, resolved on 6% polyacrylamide–7 M urea gels, and visualized by autoradiography. For sizing standards, the 5′-end-labeled substrate RNA was treated with RNase T_1 or hydrolyzed under alkaline conditions (13).

Enzymatic probing of the structure of substrate RNAs. Mung bean nuclease (Pharmacia), pancreatic RNase A, and RNase T_1 were diluted as described previously (19). Then 1×10^3 to 5×10^3 cpm of end-labeled substrate RNA and 10 μg of yeast tRNA were dried, suspended in 20 μl of 25 mM Tris-HCl (pH 7.9)–100 mM KCl–10 mM $MgCl_2$, and supplemented with enough endonuclease to obtain partial digestion. The reaction mixtures were incubated at 25°C for 30 min, and the reactions were stopped as described above.

Hybridization of COII RNA with cognate gRNA. COII mRNAs with and without the 3′ gRNA sequence (~50 fmol, 10^4 cpm) were untreated or self-annealed at 65°C for 10 min in 100 mM KCl–10 mM $MgCl_2$–25 mM Tris-HCl (pH 7.9) and then gradually cooled to 25°C. The RNA was then treated with extract or mung bean nuclease as described above.

Secondary structure modeling of preedited RNAs. Secondary structure calculations were performed with the University of Wisconsin Genetics Computer Group program FOLD (45), using updated energy values on a VAX model 8530. The following portions of the *T. brucei* maxicircle (GenBank accession no. M94286) were folded: 4032 (or 4068) to 4180 (ND7 [5′] [20]), 4764 to 4813 (ND7 [3′] [20]), 5181 (or 5208) to 5243 (COIII [14]), 6661 to 6765 and 6691 to 6765 (A6 [3]), 9730 to 9830 (MURF2, [31]), and 14448 (or 14468) to 14497 (RPS12 [26]). We also "unedited" GenBank files of several *T. brucei* edited sequences and then folded the 3′ portion of the editing domains, specifically: ND8 (GenBank M63820) 440 (or 490) to 569, ND9 (GenBank L05586) 569 (or 607) to 647, CR5 (ND3) (GenBank L26251) 346 to 465, and CR4 (GenBank U01849) 496 (or 517) to 567, with residues deleted according to Souza et al. (36), Souza et al. (37), Read et al. (27), and Corell et al. (10), respectively. We also folded sequences from unedited CYb of *T. brucei* (2), *L. tarentolae* (12), and *C. fasciculata* (33) and from unedited COII of the same organisms (12, 18, 43).

RESULTS

The sites where mitochondrial endonuclease cleaves preedited RNAs are also cleaved by heterologous single-strand-specific nucleases. Extracts of purified *T. brucei* mitochondria contain endonuclease activity that specifically cleaves in vitro-synthesized preedited CYb RNA, preedited COII RNA, and preedited COIII RNA (16). The major cleavage sites in preedited CYb RNA are in the 3′ end of the editing domain, adjacent to the first (most 3′) site that requires editing, and the major cleavage sites in preedited COII RNA also map to its small editing domain. This is shown in Fig. 2, where the editing domain is indicated by a bracket, and the sites are mapped in Fig. 3A, where the editing domain is represented by a heavy line.

To examine the specificity of the mitochondrial endonuclease, preedited CYb and COII RNAs were subjected to a variety of heterologous structure-specific endonucleases. Strikingly, mung bean nuclease, a generally recognized probe for single-stranded structure, specifically cleaves both the CYb and COII RNAs at the same major sites as the mitochondrial extract endonuclease (Fig. 2A and B; compare MBN and EXT lanes). Thus, these sites are the major available single-stranded regions of these RNA molecules. The similarity in the cleavage pattern of the extract and mung bean nuclease additionally suggests that the mitochondrial endonuclease is single strand specific. The use of the single-strand-specific and sequence-specific RNase T_1 and RNase A further confirmed that the extract endonuclease cleaves at accessible, single-stranded sites (Fig. 2A and data not shown). Figures 2A and C demonstrate that RNase T_1 (lanes T_1), like the extract endonuclease and mung bean nuclease, cleaves in the folded preedited CYb mRNA predominantly only at two adjacent positions, while it cleaves numerous G residues when the RNA is denatured (lane G). These results, plus the finding that mung bean nuclease also exhibits the same cleavage pattern as the mitochondrial nuclease on other mRNAs (see Fig. 5), including partially edited mRNAs (see Fig. 7), indicate that the *T. brucei* mitochondrial activity is a single-strand-specific endonuclease whose specificity on preedited mRNA is determined by secondary structure and does not require gRNAs. These results suggest that also in vivo, editing domains of preedited RNAs could be initially recognized by their inherent secondary structure rather than by the presence of gRNAs.

More highly resolving gels, such as that of Fig. 2C examining 5′-labeled CYb RNA, allow determination of the nature of the RNA ends produced by the *T. brucei* endonuclease. The cleavage products have electrophoretic mobilities identical to those generated by mung bean nuclease (which forms 3′-OH residues; lanes 2 and 3), and they are half a nucleotide displaced from those generated by RNase T_1 or hydroxide cleavage (which form 3′-P termini; lanes 1, 4, and 5). Because RNAs ending with a 3′ hydroxyl migrate half a nucleotide displaced from the equivalent molecules ending with a 3′ monophosphate (35), the mitochondrial endonuclease must generate 3′-OH termini, not 3′-P termini. This interpretation was confirmed by a parallel analysis using 3′-labeled CYb mRNA, in which the downstream extract cleavage products migrate as 5′-P termini, not 5′-OH termini, and was further substantiated by the observation that the 3′ end of upstream CYb cleavage product labels efficiently with pCp and RNA ligase (data not shown). 3′-OH and 5′-P termini such as these generated by the mitochondrial endonuclease are potential substrates for the trypanosome mitochondrial RNA ligase (1, 29).

Editing domains of many preedited mRNAs begin at potential single-stranded loops. The FOLD program (45) was used to examine the potential secondary structure of the preedited CYb and COII RNAs used in Fig. 2. The predicted structures (Fig. 3) agree with the nuclease data, with the major mung bean nuclease and mitochondrial extract cleavages at predicted single-stranded loops. The minor mung bean nuclease and mitochondrial extract cleavages (Fig. 2) are also in predicted single-stranded regions (Fig. 3 and data not shown), further substantiating the single-strand specificity of the mitochondrial endonuclease. (The finding that the single-strand-specific mung bean nuclease cleaves in the loop corresponding to the editing domain much more efficiently than in other regions predicted to be single stranded by the FOLD program [Fig. 2 versus 3] presumably arises because this program is not a completely accurate predictor of RNA structure.)

Could nuclease-sensitive single-stranded loops generally serve to identify editing domains? If so, then such structures should also exist in other preedited mRNAs. FOLD analyses of eight other *T. brucei* preedited mRNAs—MURF2, ND9, ND8 (CR1), ND7 (3′), ND7 (5′), CR4, RPS12 (CR6), and COIII—as well as the A6 mRNA examined by Seiwert and Stuart (30) all predict structures in which the 3′ end of the editing domain is in a single-stranded loop (e.g., Fig. 3B), like in CYb mRNA. Similar analysis of *T. brucei* CR5 preedited mRNA shows a structure where the 3′ end of the editing domain adjoins a prominent single-stranded loop, much like in COII mRNA. Furthermore, FOLD analyses of the preedited CYb and COII mRNAs of both *L. tarentolae* and *Crithidia fasciculata*, which have nucleotide sequences similar but not identical to that of *T. brucei*, all predict stem-loop structures like those in the corresponding *T. brucei* RNAs (data not shown; the potential folding of *L. tarentolae* CYb RNA was also noted in reference 32). Thus, all 16 examined preedited mRNAs are predicted to have the 3′ ends of their editing domains at a single-stranded loop, suggesting that editing do-

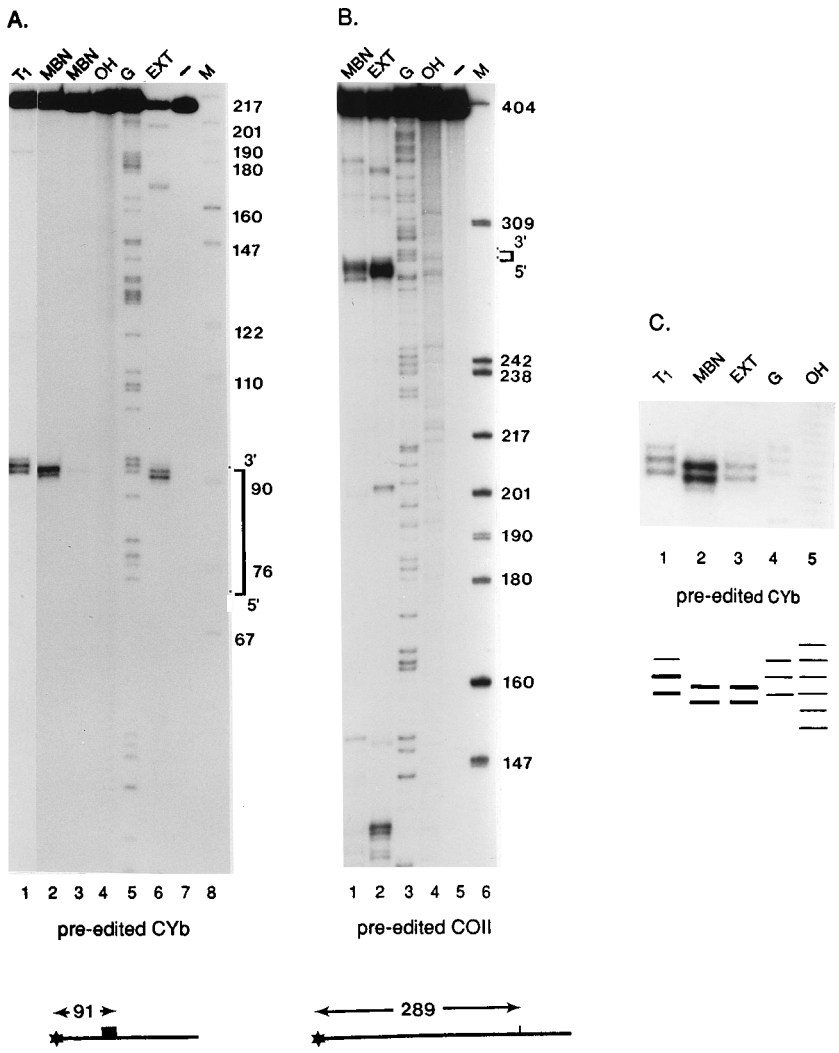

FIG. 2. Specific cleavage of preedited CYb and COII RNAs by mitochondrial extract and by single-strand-specific endonuclease. (A) 5′-end-labeled, preedited CYb RNA was treated as follows: lane 1, 0.02 U of RNase T1 per μg of RNA; lanes 2 and 3, 0.2 and 0.02 U, respectively, of mung bean nuclease per μg of RNA; lane 4, alkaline cleavage ladder; lane 5, G-specific sequencing reaction; lane 6, cleavage in the extract; lane 7, RNA with no extract. Lane 8 is a 5′-end-labeled *Hpa*II-digested pBR322 size marker. The bracket depicts the location of the editing domain. Below is diagramed the preedited CYb RNA, a 208-nt in vitro transcript in which *T. brucei* CYb sequences span nt 52 through 190 and the editing domain (heavy line) is between nt 71 and 92. The distance from the labeled 5′ end of the molecule to the 3′ end of the editing domain is shown. (B) 5′-end-labeled, preedited COII RNA was treated as follows: lane 1, 3 U of mung bean nuclease per μg RNA; lane 2, the mitochondrial extract; lane 3, G-specific sequencing reaction; lane 4, alkaline cleavage ladder; lane 5, no extract. Lane 6 is a 5′-end-labeled *Hpa*II-digested pBR322 size marker. The bracket illustrates the location of the editing domain. Below is diagramed the preedited COII RNA, a 393-nt in vitro transcript in which *T. brucei* COII sequences span nt 77 through 388 and the editing domain (heavy line) is between nt 287 and 290. The distance from the labeled 5′ end of the molecule to the 3′ end of the editing domain is shown. (C) Higher-resolution gel analysis of reactions performed analogously to those of panel A, to allow assessment of the half nucleotide displacement of fragments bearing 3′-OH and 3′-P termini. Below is a diagram of the band positions. Sizes are indicated in nucleotides.

mains in many different preedited mRNAs may be demarked by prominent single-stranded loops.

Mung bean nuclease, like mitochondrial endonuclease, can cleave edited RNAs. It was earlier reported that mitochondrial extract endonuclease did not cleave already edited CYb, COII, or COIII mRNA (16). However, further study has shown this result to be dependent on the reaction conditions. In buffer containing 10 mM DTT (restrictive conditions), edited mRNA is a very inefficient cleavage substrate for extract endonuclease whereas preedited mRNA is efficiently cleaved, causing a high specificity for preedited over edited mRNA (≥20-fold in Fig. 4, lanes 3 and 4). As the DTT concentration is reduced, however, the cleavage efficiency on the edited mRNA increases, while the cleavage efficiency on the preedited mRNA remains relatively constant (data not shown). Without added DTT (relaxed conditions), the edited and preedited mRNAs are cleaved with approximately equal efficiency by the extract endonuclease (Fig. 4, lanes 1 and 2). DTT concentrations in excess of 0.5 mM are needed to obtain a significant preference for cleavage of the preedited versus edited mRNA.

When edited CYb and COII mRNAs are treated with mung bean nuclease, they are also cleaved and at the same sites as extract endonuclease cleaves these RNAs under the relaxed assay conditions (Fig. 5). These cleavage positions are within (Fig. 5A) or adjoining (Fig. 5B) the editing domain, somewhat displaced from the cleavage sites in the preedited RNAs (compare with Fig. 2A and B). As with the preedited RNAs, both these cleavages and the minor cleavage sites outside the editing domain are at positions predicted to be single-stranded loops by FOLD analysis (data not shown). The similar cleavage pat-

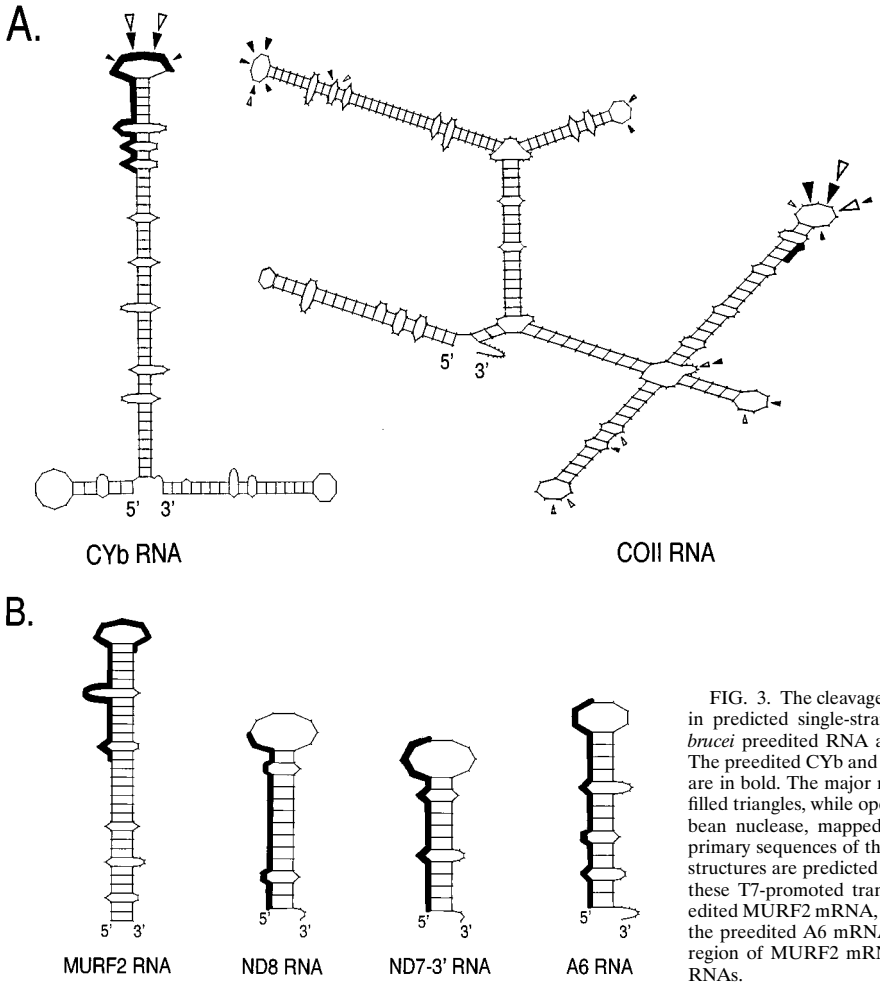

FIG. 3. The cleavage sites of preedited CYb and COII substrate RNAs occur in predicted single-stranded loops. The potential secondary structures of *T. brucei* preedited RNA as predicted by the FOLD program are illustrated. (A) The preedited CYb and COII transcripts used in this study. The editing domains are in bold. The major mitochondrial endonuclease cleavage sites are shown as filled triangles, while open triangles represent the sites that are cleaved by mung bean nuclease, mapped relative to a G track of a sequencing reaction. The primary sequences of the CYb and COII RNAs are in reference 16. Analogous structures are predicted for the natural CYb and COII preedited mRNAs as for these T7-promoted transcripts. (B) The region of the editing domain of preedited MURF2 mRNA, preedited CR1 mRNA, preedited ND7 (3′) mRNA, and the preedited A6 mRNA used in reference 30. The diagrams show an internal region of MURF2 mRNA and the 3′ portion of the CR1, ND7 (3′), and A6 RNAs.

terns of the edited mRNAs by mung bean nuclease and by mitochondrial extract under relaxed extract conditions, as well as of preedited mRNAs under all buffers conditions examined, further confirms that the extract endonuclease is single strand specific.

The difference in cleavage efficiency of edited mRNA by extract endonuclease under the stringent versus relaxed buffer conditions is not due to the DTT appreciably altering the inherent secondary structure of the edited mRNA. This was shown in control experiments in which the relative cleavage efficiency of edited versus preedited CYb mRNA by mung bean nuclease was found to be the same under both buffer conditions (data not shown). Thus, some component of the mitochondrial extract causes its difference in cleavage efficiency of edited versus preedited mRNA under the stringent conditions (Fig. 4).

Mitochondrial endonuclease does not cleave edited mRNAs duplexed with gRNA. gRNAs have been proposed to remain duplexed with the mRNA following editing (4), and they are present in the trypanosome mitochondrial extract (22, 25) (data not shown). Such duplexes between edited sequence and base-paired gRNA should be resistant to single-strand-specific endonucleases, including the editing-domain-specific endonuclease of the mitochondrial extract and mung bean nuclease. To examine this hypothesis, we used *T. brucei* COII RNA with its cognate gRNA (Fig. 6D). We identified this gRNA at the 3′ end of the COII mRNA sequence (18) by analogy with the *L. tarentolae* COII mRNA-gRNA (4). The in vitro COII transcripts used for Fig. 2 and 5 do not contain this presumptive gRNA region, but a larger COII RNA segment containing this 3′ region was cloned and transcribed in vitro to be able to assess its effect on cleavage.

Protection of COII RNA from single-strand-specific nuclease cleavage by its gRNA is shown in Fig. 6. When already edited COII mRNA containing the 3′ gRNA is allowed to self-anneal, its editing region becomes resistant to cleavage by mung bean nuclease and by the mitochondrial extract under the relaxed reaction conditions (Fig. 6A, lanes 4 and 6). Cleavage of edited COII RNA lacking the 3′ gRNA is not impaired by such self-annealing (Fig. 6B), nor is cleavage of a similarly larger preedited COII mRNA containing the 3′ gRNA (Fig. 6C). Thus, protection of edited mRNA from cleavage (Fig. 6A) requires annealing of the gRNA region with the edited region of the mRNA. Similar results were obtained with CYb mRNA and a model gRNA (data not shown). Base pairing to cognate gRNA can therefore protect edited, but not preedited, mRNA from the editing-domain-specific, single-strand endonuclease of the extract, as well as from mung bean nuclease.

To examine whether RNAs of the mitochondrial extract might be involved in creating the disparate cleavage efficiency of edited mRNA versus preedited mRNA in mitochondrial extract under the stringent reaction conditions, we pretreated

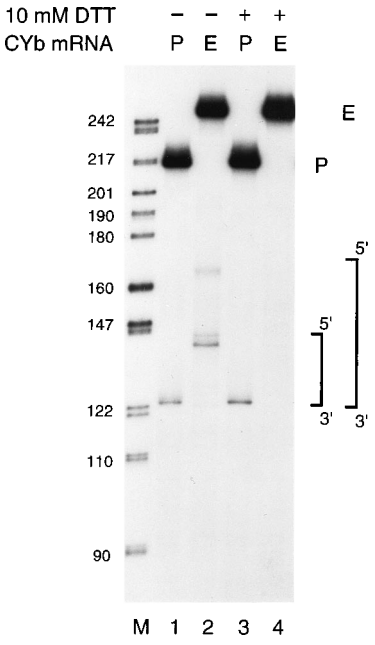

FIG. 4. Buffer conditions affect cleavage of edited mRNA in the mitochondrial extract. 3′-end-labeled preedited CYb mRNA (lanes 1 and 3) and edited CYb RNA (lanes 2 and 4) were cleaved by using 2 μl of mitochondrial extract in buffer containing 0.5 mM DTT (lanes 1 and 2; relaxed conditions) or 10 mM DTT (lane 3 and 4; stringent conditions) as detailed in Materials and Methods. Lane M is a *Hpa*II-digested pBR322 size marker. The brackets depict the locations of the editing domains on the preedited (P) and edited (E) RNAs. Extracts prepared with Triton or 3-[3-cholamidopropyl-dimethyl-ammonio]-1-propanesulfonate (CHAPS) solubilization behaved similarly. Sizes are indicated in nucleotides.

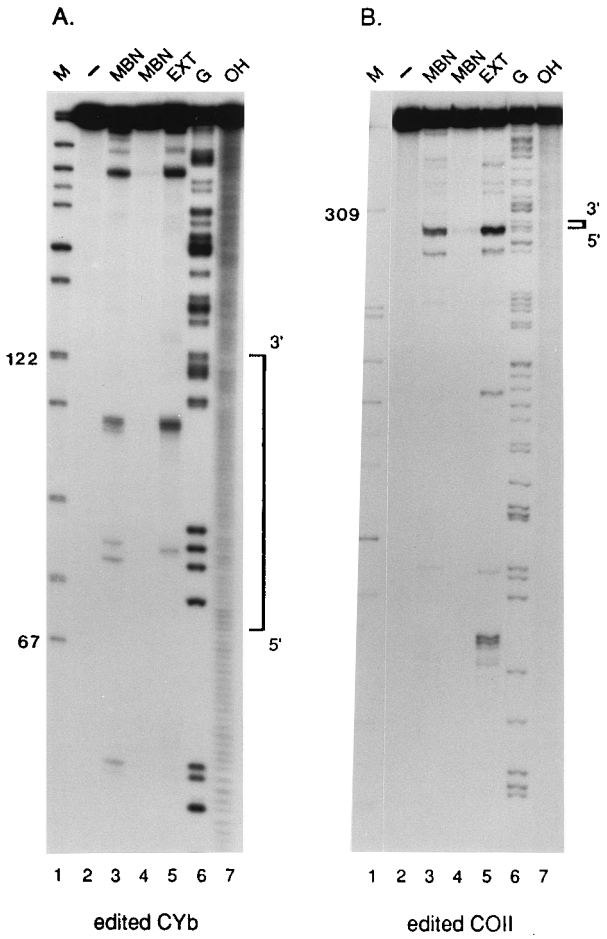

FIG. 5. Treatment of edited CYb and COII RNA with mitochondrial extract and single-strand-specific endonuclease. (A) 5′-end-labeled, edited CYb RNA was treated as follows: lane 2, no extract; lanes 3 and 4, 2 and 0.5 U, respectively, of mung bean nuclease per μg of RNA; lane 5, the CHAPS-solubilized mitochondrial extract; lane 6, G-specific sequencing reaction; lane 7, alkaline cleavage ladder. Lane 1 shows a 5′-end-labeled *Hpa*II-digested pBR322 size marker. The bracket illustrates the location of the editing domain. (B) 5′-end-labeled, edited COII RNA treated as for panel A. Sizes are indicated in nucleotides.

the extract with active micrococcal nuclease to remove available RNAs. This eliminated the preferential cleavage of preedited over edited CYb mRNA, while control reactions using calcium alone or using EGTA-inactivated micrococcal nuclease retained the preferential cleavage (data not shown). Thus, under the stringent assay conditions, RNAs of the mitochondrial extract appear to be involved in selectively affecting cleavage of edited versus preedited mRNA. This could be due to the edited RNA becoming base paired and thereby resistant to single-strand-specific endonuclease.

Nuclease analysis of aberrantly edited mRNAs. As noted above, cDNAs corresponding to numerous *T. brucei* partially edited RNAs have been cloned (12). Virtually all of these contain an aberrantly edited region between their 3′ (mature mRNA sequence) and 5′ (not yet edited) regions; it remains unclear whether they represent intermediates or dead-end byproducts of editing. We have examined the nuclease sensitivity of typical members of this set of partially edited CYb and COIII RNAs, prepared by in vitro transcription of the cloned cDNAs. Each of these RNAs shows preferential cleavage within the aberrantly edited region, principally at its 3′ end, and generally also at the 3′ end of the correctly edited region (Fig. 7). This same cleavage pattern was obtained with mung bean nuclease and with extract under relaxed or stringent conditions. Thus, the ability of these partially edited RNAs to be cleaved in extract under stringent reaction conditions is like that of preedited RNA and unlike that of fully edited RNA, and the sites of cleavage are at the positions where editing might be expected to resume if these aberrantly edited mRNAs were intermediates to obtaining fully edited RNA.

DISCUSSION

Trypanosome RNA editing has generated much interest but, as yet, few mechanistic answers. One major unanswered question is how the cycles of editing are catalyzed. It almost certainly involves gRNAs, and two favored models (Fig. 1, lower panel) envision either sets of transesterification reactions (6, 7) or sets of endonuclease and RNA ligase reactions (16, 34). A second important, but less frequently asked, question is how the editing domain is initially identified in the preedited mRNA. While recognition could theoretically be accomplished solely through base pairing of the mRNA to the 5′ end of the appropriate gRNA, analysis of partially edited *T. brucei* CYb and COIII RNAs suggests otherwise. The vast majority of these partially edited cDNAs contain segments of aberrant sequence within their editing domains (frequently starting at the 3′ end of the editing domain [11, 21, 28]). All evidence indicates that these aberrantly edited regions generally arise from faithful editing using an incorrect gRNA (9, 40, 42). Many of the 3′ aberrantly edited sequences, including those discussed in reference 11, are found in multiple independent cDNAs, and therefore they were guided, but their sequence is

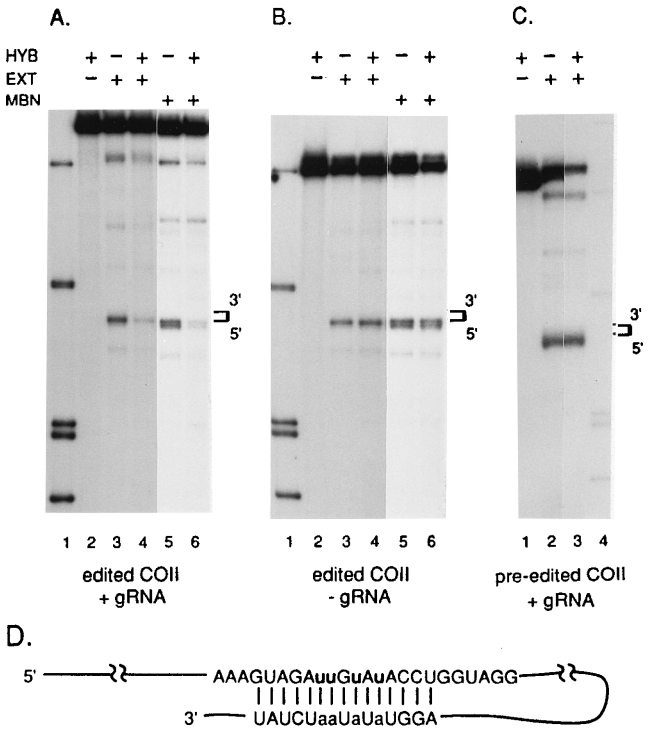

FIG. 6. Edited RNA hybridized to its gRNA is protected from cleavage. Edited COII RNA containing (A) or not containing (B) the 3′ gRNA sequences that are complementary to the edited domain, or preedited COII RNA containing the 3′ gRNA sequences (C), was not treated (−) or was treated (+) with extract (EXT) or mung bean nuclease (MBN; 3 U/μg of RNA), with (+) or without (−) prior denaturation and prehybridization (HYB). The extract reactions were done under relaxed conditions (0.5 mM DTT; see legend to Fig. 4) to allow digestion of the edited RNA. Lane 1 shows a 5′-end-labeled *Hpa*II-digested pBR322 size marker. Presumably the reason that the cleavage pattern of preedited COII mRNA is not altered by renaturation in the presence of the gRNA is that only a small fraction of the preedited COII mRNA molecules acquire the 4 bp of duplex between the gRNAs' anchor region and the preedited mRNA (see panel D), in preference to the extensively base paired structure of Fig. 2. (D) Diagram of edited COII RNA containing the 3′ gRNA hybridizing intramolecularly, with the 3′ gRNA sequences forming a duplex with the edited sequence of the editing domain. U residues added by editing are shown in lowercase. Base pairs (G · C, A · U, and G · U) are indicated with short vertical lines. The solid line represents the remaining COII RNA regions.

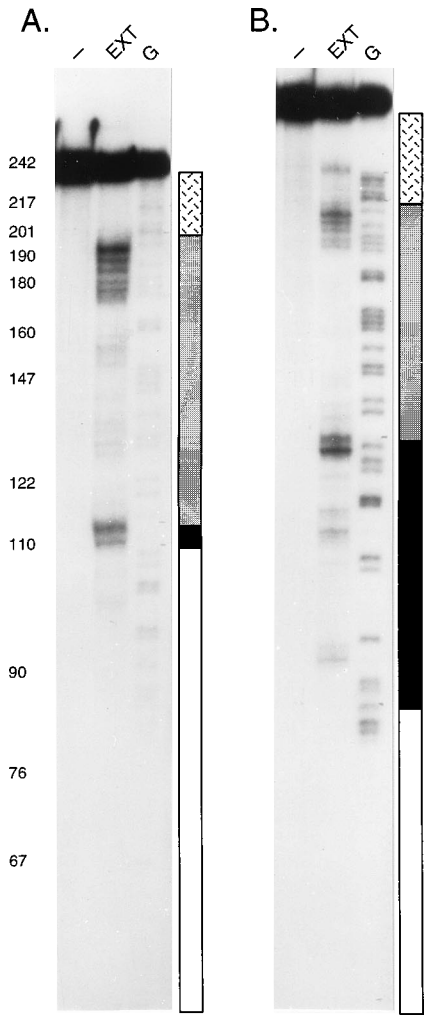

FIG. 7. Cleavage of partially edited RNAs. *T. brucei* COIII RNAs, partially edited in vivo, were transcribed in vitro from the cloned *T. brucei* cDNAs 3.1 (lanes 1 to 3) and 1.174 (lanes 4 to 6) described in reference 11. These 5′-end-labeled RNAs were not treated, treated with mitochondrial extract, or subjected to a G-specific sequencing reaction as for Fig. 2 and 5. The domains of these RNAs are represented as follows: (hatched box, region 3′ of the editing domain; grey box, already edited region; black box, aberrantly edited region; white box, 5′ preedited region.

inconsistent with arising from a misalignment of the correct gRNA, implying that they were guided by an incorrect gRNA (9, 11, 40, 42). The targeting of these wrong gRNAs specifically to the 3′ end of the normal editing domain, but not to the other vast regions of the mRNA, implies that the editing domain must be specified independently of the correct gRNA.

We previously identified an endonuclease activity in trypanosome mitochondrial extract that specifically cleaves preedited CYb, COII, and COIII mRNAs near the 3′ end of their editing domains, where editing modifications initiate in vivo, but does not cleave the edited versions of these mRNA regions (16). This endonuclease activity thus has the potential to identify the editing domains in preedited mRNA (Fig. 1, upper panel). It could also participate in the subsequent rounds of editing, in the endonuclease/RNA ligase-based editing scheme (Fig. 1, lower panel). Consistent with this latter proposal, this endonuclease and RNA ligase have been shown to be responsible for catalyzing the formation of CYb gRNA-mRNA chimeras (putative editing intermediates) in the trypanosome mitochondrial extract (24, 29). Although it remains to be proven whether editing in vivo also utilizes this enzymatic mechanism, these results on in vitro chimera formation heighten the interest in this mitochondrial endonuclease.

In this report, we show that mung bean nuclease, a heterologous and well-characterized single-strand-specific nuclease, cleaves preedited CYb and COII RNAs at the same positions as does the mitochondrial endonuclease (Fig. 2). These cleavage sites therefore demark prominent single-stranded regions. The single-strand specificity of the extract endonuclease has also been affirmed by (i) treatment with additional structure-specific nucleases (Fig. 2), (ii) computer-assisted secondary structure analysis (Fig. 3), and (iii) the generation of virtually identical cleavage patterns on already edited CYb and COII mRNAs and on partially edited COIII mRNAs, using mung bean nuclease and using extract under relaxed conditions (Fig. 5 and 7). Thus, the editing-domain-specific endonuclease of the mitochondrial extract is single strand specific, like mung bean nuclease, and this cleavage pattern is specified by the substrate RNA independent of other extract components.

The identification of the region where editing is to initiate as a prominent nuclease-sensitive single-stranded loop may not be limited to CYb and COII preedited RNAs. Computer-assisted secondary structure analysis of 14 other preedited mRNAs indicates that all can form analogous structures, with the 3′ (initiating) end of the editing domain within (13 of 14) or immediately adjacent to (1 of 14) a single-stranded loop. These examined RNAs include the CYb and COII preedited mRNAs of *C. fasciculata* and *L. tarentolae*, as well as 10 other preedited mRNAs of *T. brucei*: MURF2, ND7 (5′), ND7 (3′), ND8 (CR1), ND9, CR4, CR5, and RPS12 (CR6), COIII, and A6 (Fig. 3). Such a loop structure could serve to identify the editing domains on these other RNAs as well.

Initially, it was proposed that all of the information for editing is encoded in the secondary structure of the mRNA substrate (e.g., reference 38), but since the discovery of gRNAs (4), editing has been envisioned to be directed entirely by gRNAs, targeted to their cognate mRNAs by terminal base pairing. However the available data, presented above, indicate that (i) editing domains are identified independent of their gRNAs in vivo and in vitro and (ii) the secondary structure of the preedited RNA itself can serve to identify these editing domains in vitro. In vivo, the initial recognition of the region where editing begins may also involve RNA structure, possibly recognition by the single-strand-specific mitochondrial endonuclease.

The initial recognition of an editing domain by the mitochondrial single-strand-specific endonuclease could then serve three synergistic functions. First, its cleavage would favor breathing of the adjoining base-paired stem of the mRNA, facilitating association with the anchor region of a gRNA. (The cognate gRNA would lead to productive editing, while an incorrect gRNA would lead to aberrant editing [11]). Second, endonuclease recognition could serve to target other relevant enzymes to the editing domain. The endonuclease appears to be part of an ~20S complex (24) which also contains the RNA ligase and terminal U transferase activities (25), the other activities required in the enzymatic editing scheme. Association of the endonuclease should therefore deliver all of these complexed putative editing-related activities to the correct region of the preedited mRNA. Finally, cleavage by the endonuclease could constitute the first step in enzymatic cycles of editing. The finding that formation of gRNA-mRNA chimeras in mitochondrial extract uses endonuclease plus RNA ligase (20) is consistent with these activities being involved in RNA editing in vivo.

Single-strand specificity seems appropriate for an endonuclease involved in catalyzing cycles of RNA editing (Fig. 1). Following each cycle of editing, the sites that have already been edited would be base paired with the gRNA and thus resistant to further cleavage (Fig. 6), while the next site to be edited should be single stranded and therefore available. Base pairing with the gRNA could thereby preserve the mRNA positions that have received the correct number of U residues and ensure that editing progresses 3′ to 5′ along the mRNA.

Edited mRNAs can be either refractory or sensitive to cleavage by the mitochondrial extract, depending on the reaction conditions, but they are cleaved as well as preedited RNA by mung bean nuclease under all reaction conditions. This could be because a component of the extract can protect the edited mRNA from the nuclease action (Fig. 4 and 5 and data not shown). In vitro, gRNAs can serve this function (Fig. 6). Furthermore, destruction of RNAs in extract eliminated its marked cleavage preference for preedited versus edited CYb RNA under stringent buffer conditions (see Results). Although these results do not prove that RNAs duplex with and protect correctly edited RNAs in vivo, they affirm the feasibility of this model in the trypanosome mitochondrial extract.

The demonstration that most partially edited mRNAs cloned from *T. brucei* contained a segment of aberrantly edited sequence (11) raises the question of whether these aberrantly edited mRNAs are natural intermediates in the editing process or discarded dead-end products. Studies of a number of these mRNAs demonstrated favored nuclease cleavage, especially in the 3′ end of the aberrantly edited region and often also in the abutting correctly edited region (Fig. 7). If these RNAs were able to be reedited in vivo to yield mature functional mRNA, this is the region where the editing would be expected to resume. Furthermore, these aberrantly edited RNAs are cleaved in extract under the stringent reaction conditions, like preedited mRNA and unlike fully edited mRNA. These results are consistent with such aberrantly edited mRNAs being able to be reedited in vivo, eventually generating fully edited mRNA. Further analysis of preedited and partially edited RNAs, using mitochondrial extracts and purified components, should help elucidate the role of the extract endonuclease in the complete editing process.

ACKNOWLEDGMENTS

We thank Ken Stuart, Paul Englund, and Don Cleveland for helpful discussion and Kathy Jagger for expert assistance in preparing the manuscript.

This work was supported by PHS grants GM34231 to B.S.-W. and AI21401 to S.L.H. K.J.P. was funded by an ACS postdoctoral fellowship, and L.N.R. was funded by a Howard Hughes Medical Institute predoctoral fellowship.

REFERENCES

1. **Bakalara, N., A. M. Simpson, and L. Simpson.** 1989. The *Leishmania* kinetoplast mitochondrion contains terminal uridyl transferase and RNA ligase activities. J. Biol. Chem. **264:**18679–18686.
2. **Benne, R., B. F. De Vries, J. Van den Burg, and B. Kalver.** 1983. The nucleotide sequence of a segment of Trypanosoma brucei mitochondrial maxicircle DNA that contains the gene for apocytochrome b and some unassigned reading frames. Nucleic Acids Res. **11:**6925–6941.
3. **Bhat, G. J., D. J. Koslowsky, J. E. Feagin, B. L. Smiley, and K. Stuart.** 1990. An extensively edited mitochondrial transcript in kinetoplastids encodes a protein homologous to ATPase subunit 6. Cell **61:**885–894.
4. **Blum, B., N. Bakalara, and L. Simpson.** 1990. A model for RNA editing in kinetoplastid mitochondria: "guide" RNA molecules transcribed from maxicircle DNA provide the edited information. Cell **60:**189–198.
5. **Blum, B., and L. Simpson.** 1990. Guide RNAs in kinetoplastid mitochondria have a nonencoded 3′ oligo(U) tail involved in recognition of the pre-edited region. Cell **62:**391–397.
6. **Blum, B., N. R. Sturm, A. M. Simpson, and L. Simpson.** 1991. Chimeric gRNA-mRNA molecules with oligo(U) tails covalently linked at sites of RNA editing suggest that U addition occurs by transesterification. Cell **65:**543–550.
7. **Cech, T.** 1991. RNA editing: world's smallest introns? Cell **64:**667–669.

7a. **Clayton, D.** Personal communication.

8. **Conway, L., and M. Wickens.** 1987. Analysis of mRNA 3′ end formation by modification interference: the only modifications which prevent processing lie in AAUAAA and the poly(A) site. EMBO J. **6:**4177–4184.
9. **Corell, R. A., J. E. Feagin, G. R. Riley, T. Strickland, J. A. Guderian, P. J. Myler, and K. Stuart.** 1993. Trypanosoma brucei minicircles encode multiple guide RNAs which can direct editing of extensively overlapping sequences. Nucleic Acids Res. **21:**4313–4320.
10. **Corell, R. A., P. Myler, and K. Stuart.** 1994. Trypanosoma brucei mitochondrial CR4 gene encodes an extensively edited mRNA with completely edited sequence only in bloodstream forms. Mol. Biochem. Parasitol. **64:**65–74.
11. **Decker, C. J., and B. Sollner-Webb.** 1990. RNA editing involves indiscriminate U changes throughout precisely defined editing domains. Cell **61:**1001–1011.
12. **de la Cruz, V. F., N. Neckelmann, and L. Simpson.** 1984. Sequence of six genes and several open reading frames in the kinetoplast maxicircle DNA of Leishmania tarentolae. J. Biol. Chem. **259:**15136–15147.
13. **Donis-Keller, H., A. Maxam, and W. Gilbert.** 1977. Mapping adenines, guanines, and pyrimidines in RNA. Nucleic Acids Res. **4:**2527–2538.
14. **Feagin, J. E., J. M. Abraham, and K. Stuart.** 1988. Extensive editing of the cytochrome c oxidase III transcript in Trypanosoma brucei. Cell **53:**413–422.

15. **Hajduk, S. L., M. E. Harris, and V. W. Pollard.** 1993. RNA editing in kinetoplastid mitochondria. FASEB J. **7:**54–63.
16. **Harris, M. E., C. Decker, B. Sollner-Webb, and S. Hajduk.** 1992. Specific cleavage of pre-edited mRNAs in trypanosome mitochondrial extracts. Mol. Cell. Biol. **12:**2591–2598.
17. **Harris, M. E., D. R. Moore, and S. L. Hajduk.** 1990. Addition of uridines to edited RNAs in trypanosome mitochondria occurs independently of transcription. J. Biol. Chem. **265:**11368–11376.
18. **Hensgens, L. A. M., J. Brakenhoff, B. F. DeVries, P. Sloof, M. C. Tromp, T. H. Van Boom, and R. Benne.** 1984. The sequence of the gene for cytochrome c oxidase subunit I, a frameshift containing gene for cytochrome c oxidase subunit II and seven unassigned reading frames of Trypanosoma brucei mitochondrial maxicircle DNA. Nucleic Acids Res. **12:**7327–7344.
19. **Knapp, G.** 1989. Enzymatic approaches to probing of RNA secondary and tertiary structure. Methods Enzymol. **180:**192–212.
20. **Koslowsky, D. J., G. J. Bhat, A. L. Perrollaz, J. E. Feagin, and K. Stuart.** 1990. The MURF3 gene of T. brucei contains multiple domains of extensive editing and is homologous to a subunit of NADH dehydrogenase. Cell **62:**901–911.
21. **Koslowsky, D. J., G. J. Bhat, L. K. Read, and K. Stuart.** 1991. Cycles of progressive realignment of gRNA with mRNA in RNA editing. Cell **67:**537–546.
22. **Koslowsky, D. J., H. U. Goringer, T. H. Morales, and K. Stuart.** 1992. In vitro guide RNA/mRNA chimaera formation in Trypanosoma brucei RNA editing. Nature (London) **356:**807–809.
22a. **Peterson, P.** Personal communication.
23. **Phizicky, E. M., and C. L. Greer.** 1993. Pre-tRNA splicing: variation on a theme or exception to the rule? Trends Genet. **18:**31–34.
24. **Piller, K. J., C. J. Decker, L. N. Rusché, and B. Sollner-Webb.** 1995. *Trypanosoma brucei* mitochondrial guide RNA-mRNA chimera-forming activity cofractionates with an editing-domain-specific endonuclease and RNA ligase and is mimicked by heterologous nuclease and RNA ligase. Mol. Cell. Biol. **15:**2925–2932.
25. **Pollard, V. W., M. E. Harris, and S. L. Hajduk.** 1992. Native mRNA editing complexes from Trypanosoma brucei mitochondria. EMBO J. **11:**4429–4438.
26. **Read, L. K., P. J. Myler, and K. Stuart.** 1993. Extensive editing of both processed and preprocessed maxicircle CR6 transcripts in Trypanosoma brucei. J. Biol. Chem. **267:**1123–1128.
27. **Read, L. K., K. D. Wilson, P. J. Myler, and K. Stuart.** 1994. Editing of Trypanosoma brucei maxicircle CR5 mRNA generates variable carboxy terminal predicted protein sequences. Nucleic Acids Res. **22:**1489–1495.
28. **Riley, G. R., R. A. Corell, and K. Stuart.** 1994. Multiple guide RNAs for identical editing of Trypanosoma brucei apocytochrome b mRNA have an unusual minicircle location and are developmentally regulated. J. Biol. Chem. **269:**6101–6108.
29. **Rusché, L. N., K. J. Piller, and B. Sollner-Webb.** 1995. Guide RNA-mRNA chimeras, which are potential RNA editing intermediates, are formed by endonuclease and RNA ligase in a trypanosome mitochondrial extract. Mol. Cell. Biol. **15:**2933–2041.
30. **Seiwert, S. D., and K. Stuart.** 1994. RNA editing: transfer of genetic information from gRNA to precursor mRNA in vitro. Science **266:**114–117.
31. **Shaw, J. M., J. E. Feagin, K. Stuart, and L. Simpson.** 1988. Editing of kinetoplastid mitochondrial mRNAs by uridine addition and deletion generates conserved amino acid sequences and AUG initiation codons. Cell **53:**401–411.
32. **Simpson, A. M., N. Bakalara, and L. Simpson.** 1992. A ribonuclease activity is activated by heparin or by digestion with proteinase K in mitochondrial extracts of Leishmania tarentolae. J. Biol. Chem. **267:**6782–6788.
33. **Sloof, P., J. van den Burg, A. Voogd, and R. Benne.** 1987. The nucleotide sequence of a 3.2 kb segment of mitochondrial maxicircle DNA from Crithidia fasciculata containing the gene for cytochrome oxidase subunit III, the N-terminal part of the apocytochrome b gene, and a possible frameshift gene. Nucleic Acids Res. **15:**51–65.
34. **Sollner-Webb, B.** 1991. RNA editing. Curr. Opin. Cell Biol. **3:**1056–1061.
35. **Sollner-Webb, B., and R. H. Reeder.** 1979. The nucleotide sequence of the initiation and termination sites for rRNA transcription in Xenopus laevis. Cell **18:**485–499.
36. **Souza, A. E., P. J. Myler, and K. Stuart.** 1992. Maxicircle CR1 transcripts of *Trypanosoma brucei* are edited and developmentally regulated and encode a putative iron-sulfur protein homologous to an NADH dehydrogenase subunit. Mol. Cell. Biol. **12:**2100–2107.
37. **Souza, A. E., H. H. Shu, L. K. Read, P. J. Myler, and K. Stuart.** 1993. Extensive editing of CR2 maxicircle transcripts of *Trypanosoma brucei* predicts a protein in homology to a subunit of NADH dehydrogenase. Mol. Cell. Biol. **13:**6832–6840.
38. **Stuart, K.** 1989. RNA editing: new insights into the storage and expression of genetic information. Parasitol. Today **5:**5–8.
39. **Stuart, K.** 1991. RNA editing in trypanosomatid mitochondria. Annu. Rev. Microbiol. **45:**327–344.
40. **Sturm, N. R., D. A. Maslov, B. Blum, and L. Simpson.** 1992. Generation of unexpected editing patterns in Leishmania tarentolae mitochondrial mRNAs: misediting produced by misguiding. Cell **70:**469–476.
41. **Sturm, N. R., and L. Simpson.** 1990. Partially edited mRNAs for cytochrome b and subunit III of cytochrome oxidase from Leishmania tarentolae mitochondria: RNA editing intermediates. Cell **61:**871–878.
42. **Sturm, N. R., and L. Simpson.** 1990. Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit III mRNA. Cell **61:**879–884.
43. **van der Speck, H., G.-J. Arts, J. van den Burg, P. Sloof, and R. Benne.** 1989. The nucleotide sequence of mitochondrial maxicircle genes of Crithidia fasciculata. Nucleic Acids Res. **17:**4876.
44. **Walbot, V.** Personal communication.
45. **Zuker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9:**133–147.