Specific Cleavage of Pre-Edited mRNAs in Trypanosome Mitochondrial Extracts

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RNA editing in *Trypanosoma brucei* is a posttranscriptional processing event that results in the addition and deletion of uridine residues within several mitochondrial mRNAs. We have examined reactions involving pre-edited precursor RNAs in vitro. In this study, we report specific cleavage of pre-edited cytochrome *b* (CYb), cytochrome oxidase subunit II (COII), and cytochrome oxidase subunit III (COIII) mRNAs when incubated with *T. brucei* mitochondrial extracts. The pre-edited CYb RNA was cleaved near the 3'-most uridine addition sites, within the region where editing would be expected to commence. Pre-edited COII mRNA was similarly cleaved adjacent to its small editing domain, while pre-edited COIII RNA was cleaved at multiple sites in the region where uridine addition and deletion occurs in vivo. In contrast, edited versions of CYb, COII, and COIII RNAs were not cleaved within the editing domains. Such differential cleavage of the edited and pre-edited forms of these mRNAs suggests either a direct involvement in RNA editing or involvement in another aspect of mitochondrial gene expression requiring cleavage of pre-edited RNAs.

RNA editing in kinetoplastid mitochondria involves the addition and, to a lesser degree, deletion of uridine (U) residues at defined sites within several mRNAs (reviewed in references 3, 11, and 24). RNA editing is posttranscriptional, involving the modification of pre-edited precursor RNAs (16). Editing appears to be functionally important in the correction of a frameshift mutation in the cytochrome oxidase subunit II (COII) mRNA (4), in generation of initiation codons near the 5' ends of cytochrome *b* (CYb) and maxicircle unidentified reading frame 2 mRNAs (13, 14), and in creation of nearly the entire coding regions of the cytochrome oxidase subunit III (COIII), ATPase subunit 6, and NADH dehydrogenase subunit 7 mRNAs (5, 12, 17). The variation in the degree to which different mRNAs are edited is extreme. For example, COII mRNA has only four U's added, while completely edited COIII mRNA contains 558 added U's and has 40 U's deleted (11).

The information required for formation of edited mRNAs from pre-edited precursors is found in small (~70-nucleotide [nt]) guide RNAs (gRNAs) (6). gRNAs from maxicircle (6) and minicircle (21, 26) mitochondrial DNAs exist in the steady-state RNA population, but their precise role in editing is unknown.

Based on the structure of gRNA and the presence of mitochondrial terminal uridylyl transferase (TUTase) and RNA ligase activities, Blum et al. (6) proposed the following mechanism for RNA editing. First, a gRNA hybridizes to mRNA sequences 3' to an editing site. An endoribonuclease activity then cleaves the pre-edited RNA once at the site where the complementarity between the pre-edited mRNA and gRNA ends. The TUTase and RNA ligase activities then insert the U residues that are directed by the gRNA and rejoin the RNA chain. Decker and Sollner-Webb (10) have proposed an alternative model for RNA editing that involves

more random U additions within defined regions of the pre-edited RNA. Homology between correctly edited sequences and the gRNA sequence could protect the correctly edited sites of the mRNA from further U insertion or deletion. Both models involve endoribonuclease, U transferase, and RNA ligase activities. Nonspecific TUTase and RNA ligase activities have been detected in the mitochondria of *Leishmania tarentolae* (2). Similar activities also exist in *Trypanosoma brucei*. No endonuclease activity displaying the appropriate specificity for this editing mechanism has been reported.

As an alternative, Cech (8) proposed that editing could occur via two transesterification steps akin to RNA splicing reactions. In such a mechanism, the 3' hydroxyl of a gRNA would attack the phosphodiester bond at an editing site. This creates a chimeric RNA molecule consisting of the gRNA covalently linked to a 3' fragment of the mRNA being edited. In the second step, the 3' hydroxyl of the 5' mRNA fragment attacks a phosphodiester bond in the poly(U) tail of the gRNA portion of the chimera, generating the free gRNA with a shorter U tail and a mRNA with additional U's added at the editing site. gRNA-mRNA chimeras have been detected by polymerase chain reaction (PCR) and Northern (RNA) blot analyses (7), but nothing is known about their biosynthesis.

To learn more about the processing and expression of edited mRNAs, we have examined reactions involving pre-edited RNAs in vitro. In this study, we report cleavage at the pre-edited regions of COII, COIII, and CYb substrate RNAs in trypanosome mitochondrial extracts. Edited versions of COIII and CYb RNAs were not substrates for cleavage, nor was the editing domain of edited COII mRNA. It is likely that specific cleavage of the pre-edited versions of mitochondrial mRNAs is involved in their processing or expression.

MATERIALS AND METHODS

Preparation of *T. brucei* mitochondrial extracts and in vitro endonuclease reactions. Mitochondria from exponentially

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growing procyclic trypanosomes were prepared essentially as described previously (16). Mitochondria from 1 liter of procyclic culture (2×10^{10} cells), containing about 12 mg of mitochondrial protein, were resuspended in 1 ml of 25 mM Tris-HCl (pH 7.9)–10 mM magnesium acetate–1 mM EDTA–60 mM KCl–0.5 mM dithiothreitol–10% glycerol–1 mM ATP. Mitochondria were incubated at 25°C for 20 min to allow depletion of endogenous GTP, CTP, and UTP to levels that are unable to support transcription (16); 10% Triton X-100 in sterile H_2O was then added to a final concentration of 0.5%. After 2 min of gentle mixing, the solubilized mitochondria were placed back at 4°C and centrifuged at 10,000 × g in a microcentrifuge for 5 min, and the supernatant was assayed for endonuclease activity. Aliquots of this mitochondrial extract were stored at −70°C for up to 2 months without detectable loss in activity.

Endonuclease cleavage reaction mixtures contained 1 to 5 fmol, (1×10^4 to 5×10^4 cpm) of radiolabeled substrate RNA and 2 to 19 μl of mitochondrial extract depleted of endogenous nucleotides, plus a sufficient volume of 25 mM Tris-HCl–10 mM magnesium acetate–1 mM EDTA–60 mM KCl–0.5 mM dithiothreitol–10% glycerol–1 mM ATP to bring the final volume to 20 μl. Heparin, when included in the reaction mixture as indicated in the figure legends, was at a concentration of 5 μg/ml (2). Reaction mixtures were incubated at 25°C for 60 min unless otherwise specified; 200 μl of 40 mM Tris-HCl (pH 8.0)–50 mM NaCl–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–50 μg of proteinase K per ml was added to stop the reaction. This mixture was further incubated for 15 min at 25°C. Samples were extracted once with phenol-chloroform (1:1) and once with chloroform; 1 μg of yeast tRNA, 20 μl of 3 M sodium acetate, and 570 μl ethanol were added to precipitate the reaction products. Precipitated RNAs were recovered by centrifugation, washed once with 70% ethanol, and run on 6, 7, or 8% polyacrylamide–8 M urea gels. Radiolabeled RNAs were visualized by autoradiography.

Synthesis of substrate RNAs. Clones containing edited and pre-edited COIII and CYb sequences are described by Decker and Sollner-Webb (10). A clone containing the COII frameshift editing site as well as 200 nt of upstream sequence and 100 nt of downstream sequence was prepared by PCR (22) amplification of mitochondrial DNA, using the following primers:

COII5C, 5'-GGTTTTCAGTGATATTGGG-3'

COII3, 5'-GGCATAAAACCGTGTAATAC-3'

Thirty cycles of 1 min at 94°C, 2 min at 37°C, and 1 min at 72°C were performed. The COII PCR product was phosphorylated with T4 polynucleotide kinase (Bethesda Research Laboratories [BRl]) and ATP according to the vendor's recommendations. The phosphorylated PCR product was then blunt end ligated with T4 DNA ligase (BRL) into pBluescript KS$^+$ (Stratagene) vector digested with SmaI and EcoRV to create pCOII-pre. pCOII-ed was created by the same procedure except that first-strand cDNA, oligo(dT) primed from trypanosome mitochondrial RNA, instead of mitochondrial DNA was used. Oligo(dT)-primed first-strand cDNA of mitochondrial RNA was synthesized as described previously (19). Positive clones were characterized by sequencing.

For riboprobe synthesis, all plasmids were digested with HindIII, and T3 RNA polymerase (BRL) was used according to the manufacturer's recommendations. Uniformly labeled substrate RNAs were synthesized by performing the riboprobe synthesis reaction in the presence of 10 μCi

[α-^{32}P]GTP (400 Ci/mmol) and 500 μM each of the other deoxynucleoside triphosphates (NTPs) in a 20-μl reaction. 5'-end-labeled substrate RNAs were synthesized by first treating nonradiolabeled RNA with calf intestinal phosphatase (Boehringer Mannheim) and then labeling with T4 kinase (BRL) and [γ-^{32}P]ATP (3,000 Ci/mmol). Substrate RNAs were 3' end labeled with 3',5'[3'-^{32}P]cytidine bisphosphate (3,000 Ci/mmol) and T4 RNA ligase (BRL).

All substrate RNAs were gel purified on 4% acrylamide–8 M urea sequencing gels prior to use. Radiolabeled RNAs were visualized by autoradiography, and nonradioactive RNAs were visualized by UV shadowing. The appropriate regions of the gel were excised and eluted overnight in 0.5 M ammonium acetate–10 mM Tris-HCl (pH 8.0)–10 mM EDTA–0.5% SDS. The eluted RNA was extracted with equal volumes of phenol-chloroform (1:1) and with chloroform and then recovered by ethanol precipitation.

Primer extension and RNA sequence mapping of cleavage sites. Endonuclease reactions were performed by using 0.5 μg of nonradioactive COII substrate RNA, and the reaction products were recovered as described above. This RNA was primer extended by using the COII3 oligonucleotide and Moloney murine leukemia virus reverse transcriptase (BRL) as described previously (19). 5'- and 3'-end-labeled pre-edited COIII RNA was sequenced by using the base-specific endoribonucleases T_1 (G specific), U2 (A specific), PhyM (A+U), and *Bacillus cereus* (C+U) according to the supplier's instructions (Pharmacia). The RNA sequencing reactions and extract cleavage products were resolved side by side on polyacrylamide–8 M urea sequencing gels.

RESULTS

Endonuclease cleavage of pre-edited CYb, COII, and COIII RNAs in *T. brucei* mitochondrial extracts. The complexity of processing and turnover of trypanosome mitochondrial RNA suggests that the mitochondria may contain multiple endoribonuclease activities (23). To detect endoribonuclease activities which may be involved in mitochondrial gene expression, we incubated pre-edited and edited substrate RNAs with extracts of trypanosome mitochondria.

For these studies, we used pre-edited substrate RNAs containing the editing regions from CYb, COII, and COIII mRNAs, transcribed by T3 RNA polymerase from pBluescript vector sequences (Fig. 1; see Fig. 5 for sequences). The CYb-pre substrate RNA contains the entire CYb mRNA editing region, to which 34 U residues are to be added, plus flanking CYb sequence that is not edited. COIII-pre contains the 3' one-third of the editing domain of that transcript plus the 3' terminus of COIII RNA, which is not edited. This pre-edited mRNA region has 54 U addition sites to which a total of 136 U's are to be added and a single U deletion site. The COII-pre substrate RNA contains the small frameshift editing region of COII, to which a total of four U residues are to be added at three adjacent sites, plus a large segment of flanking COII sequence that is not edited.

Radioactively labeled pre-edited CYb, COII, and COIII substrate RNAs were incubated with Triton X-100 mitochondrial extracts under conditions which were optimal for U addition to pre-edited RNAs in isolated mitochondria (16). Fragments from apparent endonuclease cleavage were generated. To optimize the extent of cleavage, reactions were performed by using extracts that were prepared from mitochondria that had been depleted of endogenous nucleotides (16) or to which 5 μg of heparin per ml was added to inhibit mitochondrial TUTase activity (2). We reasoned that inhibi-
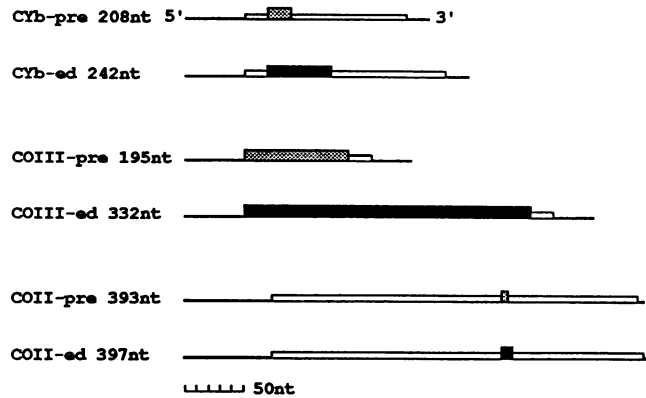
FIG. 1. Pre-edited and edited CYb, COIII, and COII substrate RNAs. Trypanosome mitochondrial RNA sequences are boxed; polylinker sequences included in the RNAs are represented by lines; sequences not modified by editing are represented by open boxes; pre-edited and edited sequences are shown as taller boxes shaded gray for pre-edited sequences and black for edited sequences.

tion of mitochondrial TUTase would minimize any alteration of the length of cleavage products by terminal U addition. Identical results were obtained whether TUTase activity was inhibited by nucleotide depletion or by heparin addition (15). Cleavage was also seen in the absence of heparin or nucleotide depletion. The amount of cleavage products was consistently reduced in these reactions, although there was considerable variation in the cleavage activity with different mitochondrial preparations (data not shown). Following the reaction, the RNA was recovered and resolved on sequencing gels (Fig. 2). To definitively map any observed cleavage sites, the analysis was performed in three complementary ways, using substrate RNAs that were 5' end labeled, 3' end labeled, and uniformly labeled.

CYb-pre RNA (208 nt) was cleaved in one specific region by the mitochondrial extract (Fig. 2A). Using 5'-labeled substrate RNA, the cleavage is seen at ~90 nt from the 5' end of the RNA (lane 5). This mapping was confirmed by using 3'-labeled RNA, for which cleavage is seen at ~120 nt from the 3' end (lane 7). This is precisely the region where editing is expected to initiate in CYb RNA in vivo, at the 3' end of the editing domain. The uniformly labeled RNA yields both the ~90- and ~120-nt products (lane 6). The fact that the 5'-end-labeled, uniformly labeled, and 3'-end-labeled RNAs yield self-consistent data indicates that the mitochondrial activity detected in this assay is an endonuclease.

COIII-pre RNA (195 nt), which contains over 50 U addition sites distributed over the central portion of the molecule, becomes cleaved at several positions, virtually all within the editing domain of the COIII RNA (Fig. 2B). There are three major regions of cleavage, each having a number of individual cutting sites. The most frequently cut region maps 40 to 60 nt from the 3' end of the molecule (lane 7), again in the region where editing initiates in vivo, at the 3' end of the editing domain. However, COIII-pre also shows significant cutting at sites ~105 and ~130 nt from the 3' end of the molecule (lane 7), further within the editing domain. The 5'- and the 3'-end-labeled and the uniformly labeled RNAs again provide complementary data.

COII-pre RNA (393 nt) also showed cleavage in the region of the editing domain (Fig. 2C). This generated a ~280-nt 5' fragment (lane 4) and a ~118-nt 3' fragment (lane 6). The 118-nt product is only weakly seen in these reactions,

probably as a result of overdigestion resulting in nonspecific loss of the cleavage product. The 118-nt 3' fragment is more clearly seen in Fig. 6, lane 11. The COII-pre RNA was also cleaved at two regions outside the editing domain. Cleavage at 50 to 60 nt and at ~270 nt, from the 3' end of the RNA (lane 6), resulted in the corresponding ~340- and ~130-nt 5' fragments (lane 4). The 270-nt 3' fragment is visible in longer exposures (see Fig. 6, lane 11). Finally, a ~300-nt 3'-end-labeled product (Fig. 2C, lane 6) which lacked a corresponding 5'-end-labeled product of ~100 nt was identified. The origin of this fragment is unknown.

Analysis of CYb, COIII, and COII pre-edited region cleavage. The kinetics of endonuclease activity were similar for all of the substrate RNAs (Fig. 3A). Accumulation of cleaved RNA was linear for approximately the first 30 min of incubation. Continued incubation for up to 120 min resulted in no further accumulation of cleaved RNA (15). Cleavage was also dependent on the amount of added extract (Fig. 3B). For most extract preparations, the maximal amount of cleavage was approximately 40% of the input RNA. Incomplete cleavage at higher extract concentrations suggests that only a subset of the substrate RNA assumes the correct conformation to be cleaved. Alternatively, the extract may contain an inhibitor which limits the extent of cleavage.

To further characterize the mitochondrial endoribonuclease activities, we attempted to inactivate them by preincubating the extract at temperatures from 4 to 65°C prior to addition of substrate RNAs (Fig. 3C). After a 15-min preincubation at the desired temperature, the extract was cooled on ice. Substrate RNAs were added, and the reaction continued at 25°C for 60 min. All of the endonuclease activity acting on the CYb and COIII pre-edited RNAs was sensitive to thermal inactivation between 45 and 55°C. The activities cleaving at the COII pre-edited region generating the ~118-nt 3' fragments were also sensitive to preincubation at 55°C but not at 45°C. Cleavage activities acting at sites distant from the COII pre-edited region displayed different thermal inactivation profiles (Fig. 3C). The cleavage activity resulting in the 50- to 60-nt 3' fragment is completely inactivated at 45°C (open triangles). The cleavage resulting in the 270-nt 3' fragment is activated at temperatures above 37°C (closed triangles). This finding suggests the presence of several endoribonuclease activities in these mitochondrial extracts. The editing site-specific cleavage reactions for all three RNAs had a thermal inactivation temperature of ~50°C.

Mapping of pre-edited sequence-specific cleavages on CYb, COIII, and COII substrate RNAs. Exact sites of endonuclease cleavage were further mapped by primer extension and by comparison of cleavage products with RNA sequencing ladders (Fig. 4). The positions of the cleavage sites determined relative to RNA sequencing ladders, COIII and CYb, were corrected to compensate for the 3' phosphate left by the sequencing enzymes (25). Cleavage sites on CYb-pre RNA were determined by comparison of both 5'- and 3'-end-labeled RNAs. Figure 4A shows the result obtained with use of 5'-end-labeled CYb-pre RNA (3' mapping not shown); Fig. 5A is a compilation of the mapping data. There are two major cleavage products which occur on the 3' boundary of the CYb pre-edited region. A lesser degree of cleavage was also observed extending further 5', into the CYb pre-edited region.

A more complex cleavage pattern was observed with the COIII substrate RNA. COIII-pre cleavage products were also mapped by comparing data obtained with use of both 3'-labeled (Fig. 4B and 5B) and 5'-labeled (data not shown)
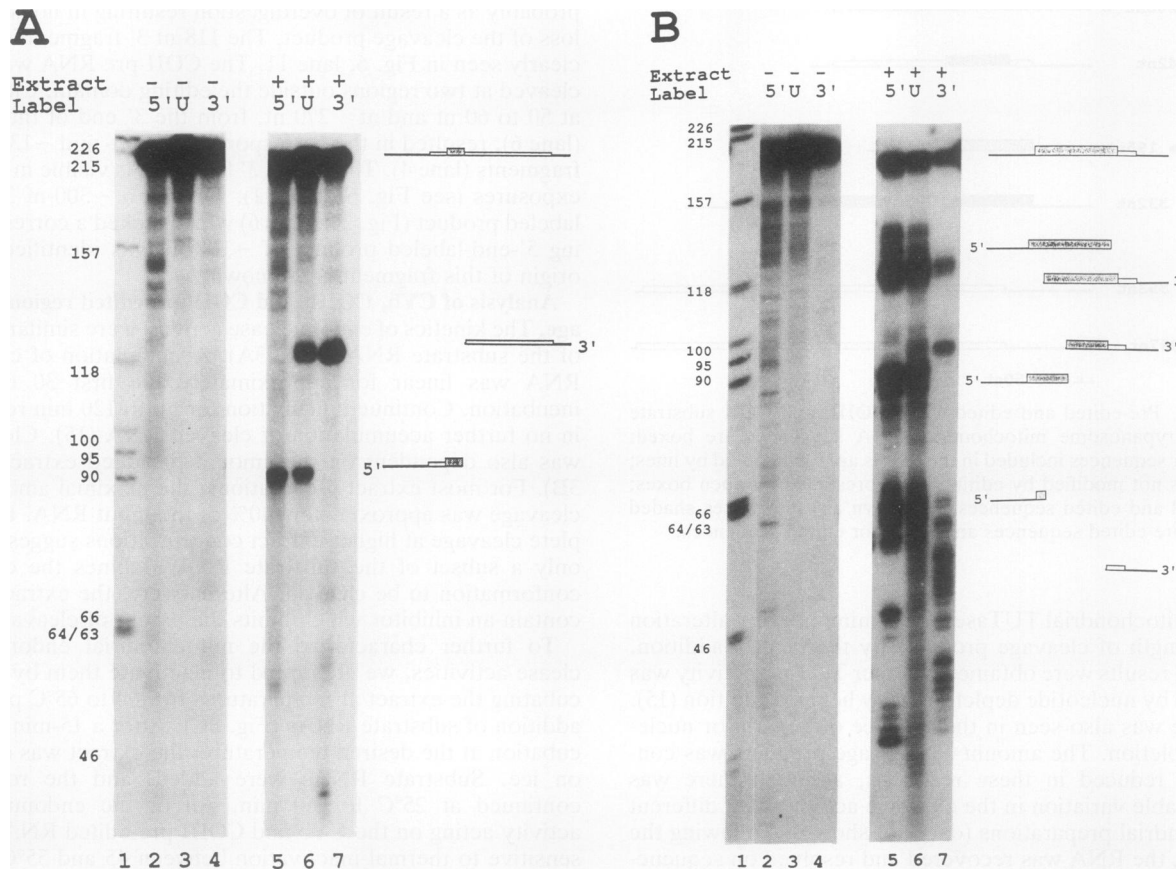
FIG. 2. Cleavage of 5' end-labeled, uniformly labeled, and 3'-end-labeled pre-edited substrate RNAs. Pre-edited substrate RNAs were either 5' end labeled (5'), 3' end labeled (3'), or uniformly labeled (U) and incubated either with (+) or without (−) mitochondrial extract. Pre-edited substrate RNAs are depicted as in Fig. 1B. Marker DNA fragments sizes are pBluescript KS⁺ digested with *Alu*I and 5' end labeled. Trypanosome mitochondrial RNA sequences are boxed, polylinker sequences included in the RNAs are represented by lines, sequences not modified by editing are represented by open boxes, and pre-edited sequences are shown as taller gray boxes. (A) CYb-pre RNA; (B) COIII-pre RNA; (C) COII-pre RNA. Only the pre-edited specific fragments are depicted. Reactions with COIII-pre contained 5 μg of heparin per ml, while reactions with COII-pre and CYb-pre used extracts depleted of endogenous NTPs.
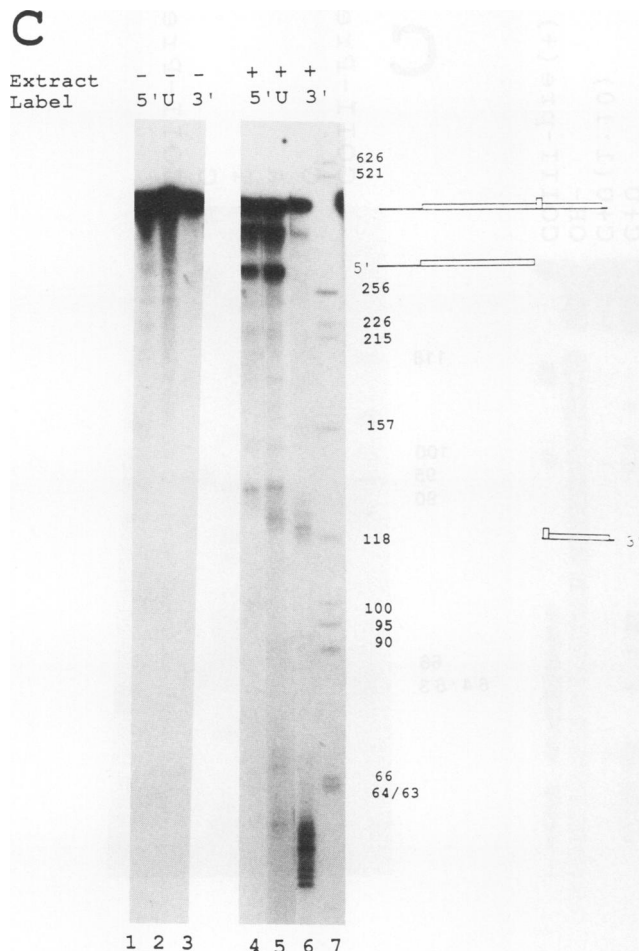
RNAs. The 3'-most U addition sites are where editing might be expected to begin, as judged from partially edited cDNA sequences (1, 10). In this region there are several cleavages, both upstream and downstream of the 3'-most U insertion site. There were no cleavages within the 35 nt of polylinker sequence at the extreme 3' end of the substrate RNA. Three cleavages were detected at the longest run of U's (three) in the substrate RNA, centered around nt 96. Several more cleavage sites were observed near the 5' boundary of the insert sequences (Fig. 5B).

Pre-edited specific COII cleavage sites were mapped by primer extension and mapping using 3'-end-labeled COII-pre (Fig. 4C and 5C). Accurate mapping using 5'-end-labeled RNA was not feasible because of the large size of the pre-edited specific 5' cleavage fragment. For primer extensions, nonradiolabeled COII-pre substrate RNA was incubated with extract, and a primer was then annealed near the 3' end of the recovered substrate RNA. These hybrids were extended with reverse transcriptase, and the extension products were compared with products of sequencing reactions using the same primer. No extension products were seen when COII-pre RNA was omitted from the reaction (15). Two major cleavages were mapped 6 and 7 nt upstream of the 5' boundary of the pre-edited region. In addition, lesser

amounts of cleavage were seen extending into the COII U addition sites.

Specificity of cleavage activities for pre-edited RNA. Cleavage at the pre-edited region of a substrate RNA may be one of the initial steps in editing or may be involved in the regulation of the steady-state levels of these mRNAs. Accordingly, the cleavage activity must recognize the pre-edited region of the substrate RNA. Edited versions of the same RNA or nonedited RNAs would not be expected to be substrates for endonuclease activities associated with these processes. Figure 6 shows a comparison of the cleavage products obtained when edited and pre-edited CYb, COIII, and COII substrate RNAs were used. No cleavage was observed when two nonedited RNAs, a 270-nt transcript from the plasmid Bluescript SK⁺ and the antisense transcript to the COII pre-edited substrate, were incubated with mitochondrial extracts (data not shown).

3'-end-labeled CYb-pre RNA was cleaved to yield a ~120-nt fragment as expected. The edited version, CYb-ed, was not cleaved. The most striking result was seen for the COIII substrate RNAs. COIII-pre RNA was again extensively cleaved. COIII-ed, which contains 154 added U's and is almost twice the size of its pre-edited counterpart, was not cut. A similar result was seen for COII-pre and COII-ed
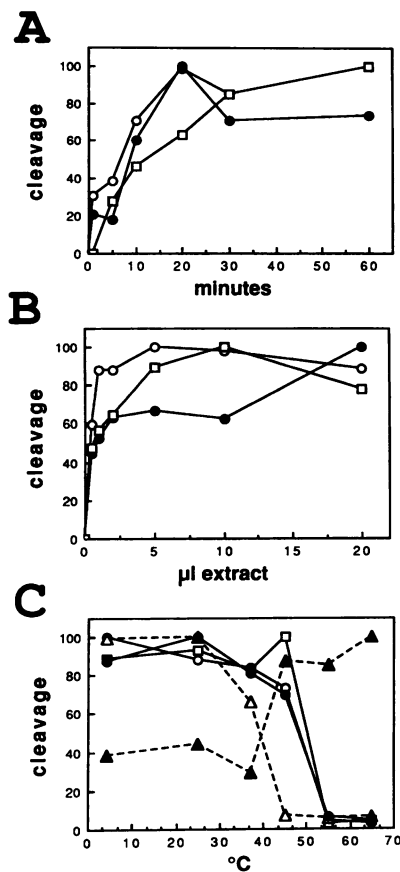
FIG. 3. Characterization of trypanosome mitochondrial endoribonuclease activities. 3'-end-labeled pre-edited substrate RNAs were cleaved, and the fragments were resolved on sequencing gels. Production of the fragments indicated below was quantified by densitometry, and the results are presented as a percentage of the maximum amount of cleavage. (A) Kinetics of endoribonuclease cleavage generating the ~120-nt CYb-pre 3' fragments (see Fig. 2) (open circles), the ~118-nt COII-pre 3' fragments (squares), and the 40- to 60-nt COIII-pre 3' fragments (filled circles). Substrate RNAs were incubated with a constant amount of mitochondrial extract (2 μl for CYb-pre and COII-pre; 10 μl for COIII-pre), and samples were taken at the indicated times. Reactions with COII-pre and COIII-pre substrate RNAs contained 5 μg of heparin per ml. (B) Titration of endoribonuclease activities cleaving at the CYb, COIII, and COII pre-edited regions. Increasing amounts of extract were added to individual cleavage reactions, and the mixtures were then allowed to incubate for 60 min. Symbols are as in panel A. (C) Thermal inactivation of pre-edited region endonuclease cleavage (solid lines) and cleavage of COII at sites distant from the pre-edited region (broken lines). Five-microliter aliquots of extract, diluted to 20 μl with reaction buffer, were preincubated for 15 min at the indicated temperature and then placed on ice. Substrate RNAs were added, and the reaction mixtures were incubated at 25°C for 60 min. The pre-edited region cleavage fragments analyzed are the same as in panel A: the ~120-nt CYb-pre fragments (open circles), the 40- to 60-nt COIII-pre fragments (filled circles), and the ~118-nt COII-pre fragments (squares). Analysis of 50- to 60-nt COII RNA 3' fragments (open triangles) and ~270-nt COII RNA 3' fragments (filled triangles) which result from cleavage at sites distant from the COII pre-edited region is also included.

RNAs. COII-pre was cut at the pre-edited region, yielding 3' fragments at about ~118 nt, while COII-ed, which differs only by the addition of four U's at the editing site, lacked these cleavages. It did, however, continue to be cleaved at sites which were distant from the pre-edited region. The ~300-nt product observed with use of 3'-end-labeled RNA was also not seen when edited COII substrate RNA was used. It may be that these products are derived from the ~118-nt pre-edited RNA-specific cleavage fragments (15).

DISCUSSION

The studies presented here demonstrate the presence of an endonuclease activity in trypanosome mitochondrial extracts that selectively cleaves pre-edited but not edited CYb, COIII, and COII RNAs (Fig. 5). The other activities believed to be involved in RNA editing, TUTase and RNA ligase, have previously been detected (2, 15), but the reported activities are nonspecific. The endoribonuclease activity that we have detected is specific, not only for pre-edited RNAs but also for its site of cleavage. The specific cleavage sites map at or adjacent to sites where U's are added to form the mature, edited mRNAs. In CYb as well as in COIII, the major cleavages are at the 3' end of the editing domain of these pre-edited RNAs. These are the regions where sequence analysis of RNA partially edited in vivo

have indicated that the editing modification process initiates (1, 10, 17, 27).

We have recently observed formation of a gRNA-mRNA chimera in vitro with use of the CYb-pre substrate RNA and
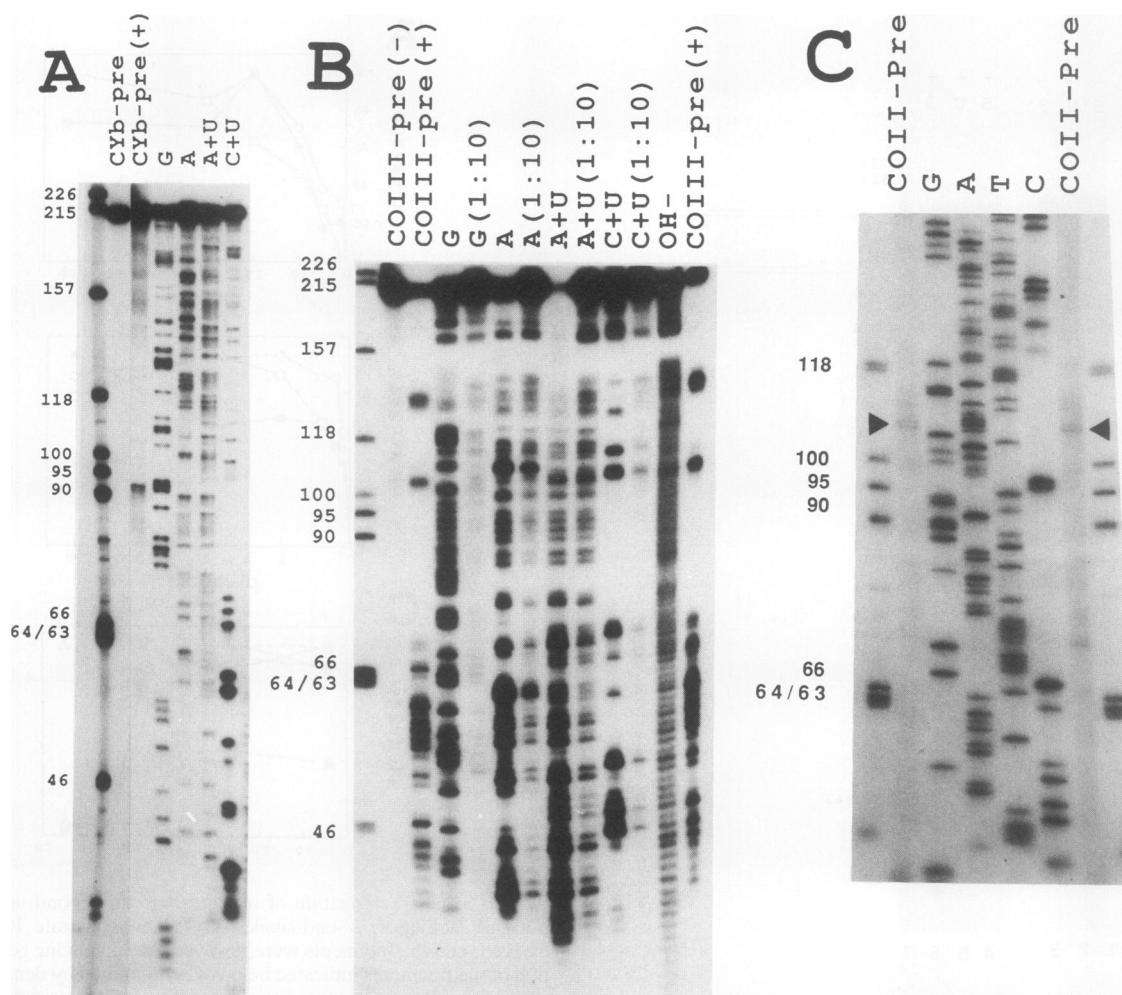
FIG. 4. Mapping of pre-edited region cleavage sites. (A) Mapping using 5'-end-labeled CYb-pre RNA. Labeled RNA was incubated either with (+) or without (−) mitochondrial extract. Extract cleavage products were resolved adjacent to cleavage products by using base-specific endoribonucleases. (B) 3'-end-labeled COIII-pre RNA digested with (+) or without (−) trypanosome mitochondrial endoribonucleases and with base-specific endoribonucleases at two dilutions. The lane marked OH− indicates an alkaline hydrolysis ladder of COIII-pre RNA. (C) Primer extension mapping of COII-pre RNA cleavage fragments. Unlabeled COII RNA was incubated with mitochondrial extracts; the RNA was recovered and hybridized with a primer directed against the 3' portion of the substrate RNA. Hybrids were extended with reverse transcriptase, and the fragments were compared with sequencing reactions using the same primer. Arrowheads mark the position of the major COII pre-edited RNA-specific cleavage sites. All reactions were performed with extracts depleted of endogenous nucleotides.

synthetic CYb gRNA (15). Consistent with the endonuclease being involved in editing is the observation that the junction between the gRNA and mRNA sequences occurs at or near the endonuclease cleavage sites. This finding suggests that the gRNA-mRNA chimeras observed in vivo could be products of the endonuclease and of the mitochondrial RNA ligase. In this case, the gRNA would be ligated onto the 5' end of the 3' cleavage fragment to generate the chimera.

It has recently been proposed that editing could be accomplished not by endoribonuclease, TUTase, and RNA ligase reactions but by two transesterification steps (7, 10). It seems likely that a reaction similar to 5' splice site hydrolysis in group I introns could also give rise to the endonuclease cleavage products that we observe. This predicts that the RNA fragments generated in vitro are not then substrates for subsequent steps in editing. The 3' cleavage fragment generated in this reaction would not be able to form a gRNA-mRNA chimeric molecule.

Cleavage of pre-edited COIII RNA appears consistent with a less processive editing process, as suggested by the structure of many cDNA clones of partially edited mRNA (1, 10, 17). Unlike the cleavage of pre-edited CYb RNA, which was limited to the 3' U addition sites, cleavage of COIII occurred within three regions. One of the regions is centered around the 3'-most U addition site. The two other regions of the RNA that are cleaved by the extract may represent portions of the RNA edited by different gRNAs (21). This possibility is consistent with the observation that the second cleavage domain is at the end of the edited region of a number of partially edited COIII RNAs (10).

Major cleavages of the pre-edited COII RNA were 6 and 7 nt 5' to the actual U addition sites. Lesser amounts of cleavage were seen extending into the COII U addition sites. While it is possible that our extract preparation lacks factors necessary for the efficient cleavage of COII RNA at the actual U addition sites, the observed cleavages upstream of

A

CYb RNA

GGGAGAGAAU UCGAGCUCGG UACCCGGGGA UCCUCUAGAG UCGACCUGCA

GGGUUAUAAA UUUUAUAUAA A$_u$A$_u$G$_{uuu}$CG uu^G_uAGA$_{uuu}$ uu^{A}uu^{A}uuuu

uuuu^{A}uu^{A}uu $_u$AGAAA$_{uuu}$G $_u$G$_{uu}$GUCUUU UAAUGUCAGG UUGUUUAUAC

AGAAUAUAUG GGGUAGGUUU UAGUUUAGGA UUUUUUAUAG CAUUGCAAAU

AAUUUGUGGA GUGUGUUUAG CUUGACGCGU GGCAUGCAAG CU

B

COIII RNA

GGGAGAGAAU UCGAGCUCGG UACCCGGGGA UCCUCUAGAG UCGACCUGCA

GAGG$_u$G$_{uuuu}$ G$_{uu}$G$_{uu}$G$_{uuu}$ A$_{uuu}$G$_{uuuu}$A $_u$GCG$_{uuu}$G$_{uu}$ u^{AA}$_{uuuuuu}$G

u^G_uA$_u$GGA$_u$A CACG$_{uuuu}$G$_u$ uuuuuu^G_uA$_u$ u^G_uG$_{uuu}$G$_{uu}$ u^A_uA$_{uu}$GACA

uuuu$^G_{uu}$GAU UUAG$_{uuu}$GA$_u$ uuuuuuu$^A_{uu}$ GCGA$_{uuu}$G$_{uu}$ u$^A_{uuuu}$GA$_u$G

uuuu^A_uG$_u$G$_u$ u^A_uG$_u$A$_{uuu}$G $_u$G$_u$G$_u$G$_u$AA$_u$ uuu$^A_{uu}$GG$_u$G $_{uuuu}$UUUAGU

^{U}G$_{uu}$GA$_{uu}$AU G$_{uu}$AA$_{uuu}$G$_u$ A$_{uu}$GGUAGUU UGUAGGAAGU UU**CCCCCCCC**

CCCCCCCCCA CGCGUGGCAU GCAAGCU

C

COII RNA

GGGAACAAAA GCUGGAGCUC CACCGCGGUG GCGGCCGCUC UAGAACUAGU

GGAUCCCCCG GGCUGCAGGA AUUCGAGGUU UUCAGUGAUA UUGGGUAUAU

UUUUUAUUGG AGAAACCACG AUAUUUAGUA AUUUAAUAUU AGAAAGUGAU

UAUUUAAUAG GAGAUUUAAG AAUAUUACAG UGUAACCAUG UAUUGACAUU

GUUAAGUUUG GUUAUUUAUA AAUUAUGAGU AUCUGCAGUA GAUGUAAUAC

ACUCAUUUAC AAUAUCAAGU UUAGGUAUAA AAGUAGA$_{uu}$G $_u$A$_u$ACCUGGU

AGGUGUAAUG AAAUAAUUUU GUUUGCUACA AAUAACGCAA CUCUUUACGG

ACAAUGUAGU GAAUUGUGUG GUGUAUUACA CGGUUUUAUG CC**UAUCA**

FIG. 5. Pre-edited RNA-specific cleavage sites on CYb-pre (A), COIII-pre (B), and COII-pre (C). Sequences present in the pre-edited RNAs are shown in uppercase letters; positions of added U's are shown in lowercase below the pre-edited RNA sequence. The single U deletion site in COIII-pre (B) is indicated by a bold uppercase U in the pre-edited RNA sequence. Polylinker sequences included in the substrate RNAs are underlined. Endonuclease cleavage sites are indicated by arrowheads above the appropriate nucleotides. Only the COII-pre cleavages that were located adjacent to the pre-edited region are depicted.
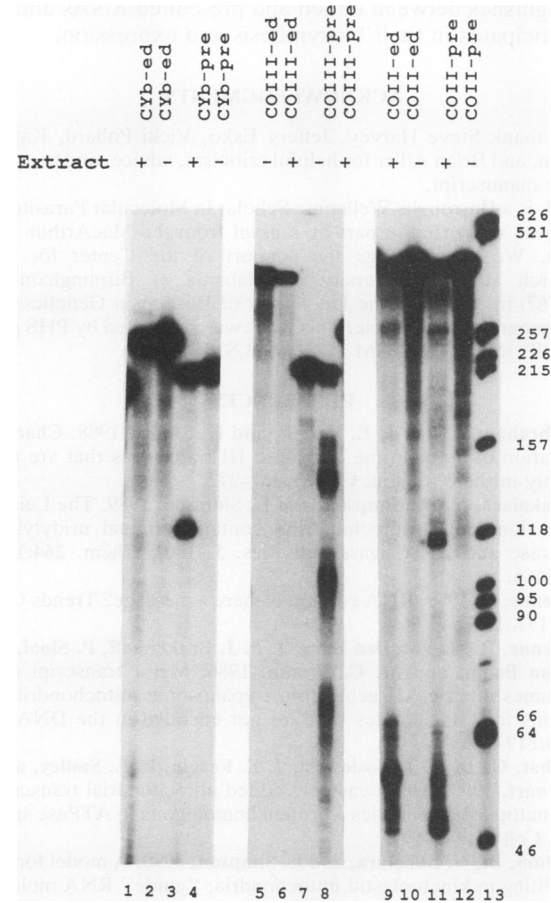

FIG. 6. Incubation of pre-edited and edited CYb, COIII, and COII RNAs with mitochondrial extracts. Lanes: 1 to 4, 3'-end-labeled edited and pre-edited CYb substrate RNAs incubated with 2 μl of trypanosome mitochondrial extract (lanes 1 and 3) or without extract (lanes 2 and 4); 5 to 8, uniformly labeled edited and pre-edited COIII substrate RNAs incubated with 10 μl of extract (lanes 6 and 8) or without extract (lanes 5 and 7) (in this case, 5 μg of heparin per ml was added to inhibit TUTase activity); 9 to 12, 3'-end-labeled edited and pre-edited COII substrate RNAs incubated with 5 μl of extract (lanes 9 and 11) or in the absence of extract (lanes 10 and 12).

the COII editing site could facilitate editing by altering the secondary structure of the pre-edited RNA. This initial cleavage might allow gRNAs to interact with the U addition sites and cause subsequent modifications to occur.

There are several possible reasons for the difference in cleavage of pre-edited versus edited RNA substrate. It is possible that the endonuclease recognizes a specific sequence or secondary structure within the pre-edited RNA which is modified by U addition or deletion. Alternatively, the pre-edited RNA might anneal to a specific gRNA, producing a structure recognized and cleaved by the mitochondrial enzyme. The edited RNA would form a perfect duplex with the gRNA and might be resistant to further cleavage.

It could also be argued that the pre-edited RNA-specific nucleases might be involved in RNA turnover. During its developmental cycle, *T. brucei* regulates the level of its mitochondrial mRNA and rRNA, in some cases up to several hundred fold (20). Steady-state levels of edited transcripts might be regulated at the level of turnover of pre-edited precursor RNAs. Specific degradation of pre-edited RNAs could thus play a role in developmental expression of the mitochondrial genome.

Another issue concerns the composition of the cleavage activities. They could be similar to endoribonucleases involved in the splicing of tRNAs (18). Alternatively, the cleavage could be mediated by RNA (9). A third possibility is that the pre-edited mRNAs are self-cleaving but require extract components for efficient cleavage. These might resemble the maturase proteins involved in self-splicing in yeast mitochondria (28). Further purification and characterization of the activity is under way to examine how it

distinguishes between edited and pre-edited RNAs and how it participates in their biosynthesis and expression.

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