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Endoribonuclease activities of *Trypanosoma brucei* mitochondria

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Abstract

RNA editing in kinetoplastids is a type of post-transcriptional processing that changes mitochondrial mRNA sequences by the addition or deletion of uridines. Multiple enzymatic activities, such as endoribonuclease and RNA ligase, are associated with this process and exist in a multienzyme complex. Endonuclease activities from *Trypanosoma brucei* mitochondrial extracts were fractionated by sequential ion exchange and gel filtration chromatography. The RNA editing specific endonuclease activity co-fractionated with in vitro editing while another endonuclease activity with a different substrate specificity, and the majority of mtRNase P activity fractionated away from the editing activity. The pH, salt, temperature, and Mg^{2+} optima of all three endonucleases were determined. All three activities are sensitive to high temperature and protease digestion. In addition, treatment with micrococcal nuclease resulted in partial disruption of the editing complex and decreased pre-cleaved in vitro insertion editing activity, suggesting that both RNA(s) and protein(s) are necessary in the intact functional complex.

Keywords: RNA editing; Endoribonuclease; RNase P; Mitochondria

1. Introduction

Kinetoplastid RNA editing entails the addition and deletion of uridines and it is catalyzed by a multiprotein complex [1–5]. This complex catalyzes the endoribonucleolytic cleavage, uridylate addition and removal, and RNA ligation steps of editing. The edited sequence is specified by guide RNAs (gRNAs) which have distinctive primary structure. The 5′ region can form a 4–14 'anchor' basepair duplex with pre-mRNA immediately 3′ (i.e. downstream) of the region that will be edited. The central portion of the gRNA contains the guiding or informational region that specifies the edited sequence. It is generally about 35 nucleotides in length and is perfectly complementary (including G/U basepairs) to the edited sequence. The 3′ region of the gRNA contains a 3′ oligo(U) tail of variable length that is added post-transcriptionally. The three regions of all gRNAs have specific functions. These include selection of the cognate pre-mRNA for editing by formation of the anchor duplex, directing the endoribonucleolytic cleavage that initiates editing at a specific editing site (ES), and specification of the edited sequence by the informational region. The oligo(U) tail may maintain the association of the gRNA with 5′ portion of the pre-mRNA during editing.

Much of the knowledge of the editing mechanism was obtained using an in vitro editing system which contained synthetic pre-mRNA, synthetic cognate gRNA, mitochondrial extract, ATP, and inorganic constituents [6–8]. This in vitro system initially utilized ATPase subunit 6 (A6) pre-mRNA and results in gRNA-specified editing at a single site. Editing required the gRNA and mitochondrial extract and the sequence of gRNA determined the number of Us that were added or deleted. Incubation of pre-mRNA with the cognate gRNA resulted in an endonucleolytic cleavage immediately 5′ to the anchor duplex leaving the phosphate at the 5′ terminus of the 3′ cleavage product and

Abbreviations: gRNAs, guide RNAs; ES, editing site; A6, ATPase subunit 6; Cyb, cytochrome b; MN, micrococcal nuclease; D, digitonin.

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the hydroxyl at the 3′ end of the 5′ cleavage product. The specificity of U insertion editing is determined at all three steps of editing [8], while specificity of deletion editing includes the U-specificity of editing exonuclease (Lawson, Igo Jr., Salavati, Stuart, in press).

Several endoribonuclease activities that differ in cleavage specificity have been described in mitochondrial extracts from *Trypanosoma brucei* and *Leishmania tarentolae* [9,10]. Three endoribonuclease activities have been described in *T. brucei* including one that cleaves at the most 3′ editing site of cytochrome b (CYb) mRNA upon inclusion of CYb gRNA with a downstream duplex [1,3,10,11]. The only endoribonuclease that has been isolated, MAR1 from *L. tarentolae* [12], cleaves pre-edited mRNAs independently of gRNAs and thus may not be involved in editing. To date, no RNA editing endonuclease gene has been clearly identified. However, two editing ligases were initially recognized by their autoadenylation activity [13] and have been shown to be components of the editing complex [14–16] and one (TbMP52) is essential for editing [16].

In this report, we describe enrichment of RNA editing-associated endonuclease activity and other endonuclease activities that did not copurify with the in vitro editing. In addition, we show by treatment of the complex by micrococcal nuclease (MN) that RNA is important for the integrity and function of the editing complex.

2. Materials and methods

2.1. Preparation of precursor substrates

The CYb pre-edited mRNA (104 nt) was synthesized by T7 RNA polymerase from a *Bam*HI linearized DNA template [11,17]. The CYb guide RNA (30 nt) that directs insertion of 2, 1, and 3 U residues at ES1, ES2 and ES3, respectively, was synthesized by T7 RNA polymerase from two complementary oligonucleotides, as previously described [2]. Pre-tRNA^{Ser} was synthesized as previously described [18]. 5′CL18, 3′CL13pp and gA6PC RNAs were synthesized as previously reported [19]. A 110 nucleotide pBluescript RNA was in vitro transcribed using *XbaI*-digested pBluescript as a template.

2.2. Assays for enzymatic activities

Endonuclease assays contained, in a final volume of 20 μl, pre-edited CYb 5′-end labeled mRNA (10 fmol), ± 1 pmol CYb guide and 5 μl of mitochondrial extract, in 10 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, (0–5) mM DTT, and 4U RNAsin. Following incubation for 40 min at 28 °C, 20 μl of 10 M urea dye were added and the samples were run on a 9% denaturing gel (7 M urea). These reaction products were analyzed on a Molecular Dynamics PhosphorImager system using Image Quant software. One unit of gRNA-dependent endoribonuclease activity is defined as the amount of the enzyme required to cleave 2% of 10 fmol of CYb pre-mRNA in 40 min under assay conditions. mtRNase P assays tested the ability of fractions to cleave ^{32}P internally labeled or 5′ labeled wild type and mutant *Schizosaccharomyces pombe* pre-tRNA^{Ser} substrates as previously reported in Ref. [18]. Precleaved insertion editing and RNA ligase reactions were performed using 5′CL18, 3′CL13pp and gA6PC RNAs as previously reported in Ref. [19].

2.3. Purification of T. brucei endonuclease activity

The endonuclease was purified from mitochondrial lysate from 12 l of log-phase procyclic *T. brucei* IsTaR 1.7a [20] by sequential SP Sepharose, Q Sepharose, and Superose-6 fractionation as previously described [21]. For digitonin or micrococcal (MN) and digitonin treatments, 0.5 l of digitonin-treated (at 90 μg mg^{-1} of mitochondrial protein) or digitonin + MN (900 U ml^{-1}) treated mitochondrial suspension was purified by Superose-6 as previously described [18]. Briefly, digitonin was incubated with mitochondrial suspension resuspended in 1 ml of 10 mM Tris pH 7.0, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 2 μg ml^{-1} Leupeptin, 1 μg ml^{-1} Pepstatin, 1 μM Pefabloc at 4 °C with gentle rotation for 15 min. The mitochondria were isolated by centrifugation at $12\,000 \times g$ for 20 min. $CaCl_2$ was added to 2 mM and the suspension was split in to two halves. MN (350 U ml^{-1}) was added to one or H_2O to the other half followed by a 30 min incubation at room temperature. EGTA (10 mM) was added to both suspensions followed by lysis with 0.5% Triton X-100 at 4 °C for 15 min.

2.4. Determination of enzyme characteristics

The optimal temperature and pH were determined from the initial velocity of the cleavage reaction over a range of temperature and pH. The extracts from the SP Sepharose column were pre-equilibrated for 2 min at the temperature of assay before the substrates were added to the assay mixtures. Aliquots were removed and the reaction was terminated by the addition of equal volume of a 10 M urea loading buffer. The optimal temperature was determined in 10 mM Tris (pH 7.0), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, and 0.2 U μl^{-1} RNAsin. The optimal pH was determined at 28 °C in a buffer containing 10 mM $MgCl_2$ and 50 mM Tris–HCl, and 50 mM KCl over a range of pH 6.0–8.5. The effects of $MgCl_2$ and KCl were determined using diluted extract in 10 mM Tris pH 7.0 buffer so that the maximum concentrations of $MgCl_2$ and KCl were 30 and 200 mM, respectively.

Table 1
Purification of endoribonucelase associated with RNA editing from *T. brucei*

Material (column)	Volume (ml)	Total Protein (mg)	Total activity ($\times 10^3$, U)	Specific activity (U mg^{-1})[a]	Purification (Fold)
Crude extract	40	120	40	333	1
Sp-seph. (F9–19)	22	8	48	6000	18
Mono-Q (F11–20)	10	1	40	40 000	120
Superose 6 (F19–22)	6	0.1	21	210 000	630

[a] 1Unit = endoribonuclease required to convert 2% of 10 fmol of CYb pre-mRNA in 40 min under assay conditions.

2.5. Extraction of RNA, 3′-end labeling, and dot blot hybridization analysis

Fractions from the Superose-6 column were pooled and subjected to RNA extraction by the acid guanidium thiocyanate–phenol–chloroform extraction method [22]. RNA species present in active fractions were end labeled as previously described [23]. Dot blot analysis was performed using the isolated RNA and 10 ng of total mitochondrial RNA essentially as described in Ref. [24]. RNA was isolated from pooled Superose 6 fractions (19–24) and hybridized with the MURF4.1 oligonucleotide probe (complementary to 3′ A6 mRNA sequence that is not edited, shows pre-edited A6 as well as partially edited A6 mRNA); MURF4.14 oligonucleotide probe (complementary to 5′ edited A6 mRNA sequence, shows fully and partially edited A6 mRNA); gA6[14]gRNA oligonucleotide probe (shows the presence of gRNA); and BOXB oligonucleotide probe (5′-GGATTTGAACC-3′, complementary to BOXB of tRNAs) as previously described in Ref. [25]. Hybridization was performed in Rapid-Hyb buffer (Amersham) according to the manufacturer's recommendation.

3. Results

RNA editing-associated endoribonuclease activity was enriched over 600-fold by serial chromatography as summarized in Table 1. Endoribonuclease activity that cleaved both 5′ ^{32}P labeled A6 and CYb pre-mRNAs at their first (most 3′) ES as directed by their cognate gRNAs co-fractionated with in vitro deletion editing (Fig. 1 and [21]). The apparent peak of endonuclease activity is one fraction ahead of deletion editing but the peak of endonuclease activity must include chimera forming activity since this requires endonuclease activity [7] and these combined activities coincide with the peak of editing activity. This activity which is not sensitive to DTT (data not shown) eluted from SP Sepharose cation exchange columns at 200–300 mM KCl, while the endoribonuclease activity that cleaved CYb gRNA at ES2 and ES3 in the absence of CYb gRNA eluted at 100–150 mM KCl (Fig. 1A). The co-fractionation of the gRNA-dependent cleavage activity suggests that this activity has a role in editing. The role of the other endonuclease activity is not known. RNase P activity co-eluted with the gRNA directed endonuclease and in vitro editing activities from SP Sepharose (Fig. 1A) and eluted at lower KCl concentration from Q Sepharose anion exchange columns, but with an overlapping profile (Fig. 1B). Most gRNA specific endoribonucleolytic activity eluted at 1600 kDa from Superose 6 columns but a small

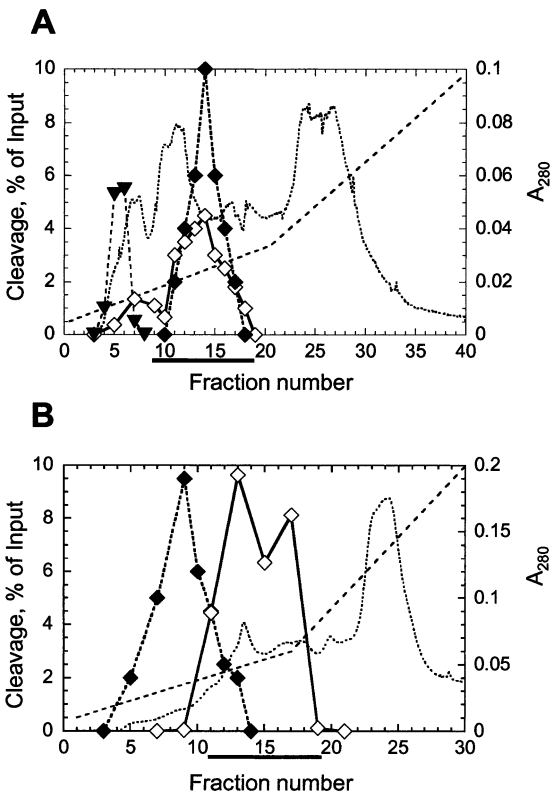

Fig. 1. Ion exchange fractionation of mitochondrial endoribonuclease activities. gRNA-dependent endonucleolytic cleavage of A6 or Cyb pre-mRNAs at ES1 (open diamonds) or cleavage of 5′ labeled Cyb gRNA at ES2 in the absence of gRNA (triangles). Cleavage of internally labeled *S. pombe* pre-tRNA^{ser} at the site cleaved by RNase P activity (closed diamonds). See Section 2 for details. Fractions containing in vitro editing activity are indicated by the underlines, A_{280} by dotted lines, and the KCl elution gradient profile by dashed lines (the KCl concentration in M is the value on the left hand *y*-axis divided by 10). (A) SP Sepharose elution profile of mt extract. (B) Q Sepharose elution profile of pooled fractions 9–19 from A.

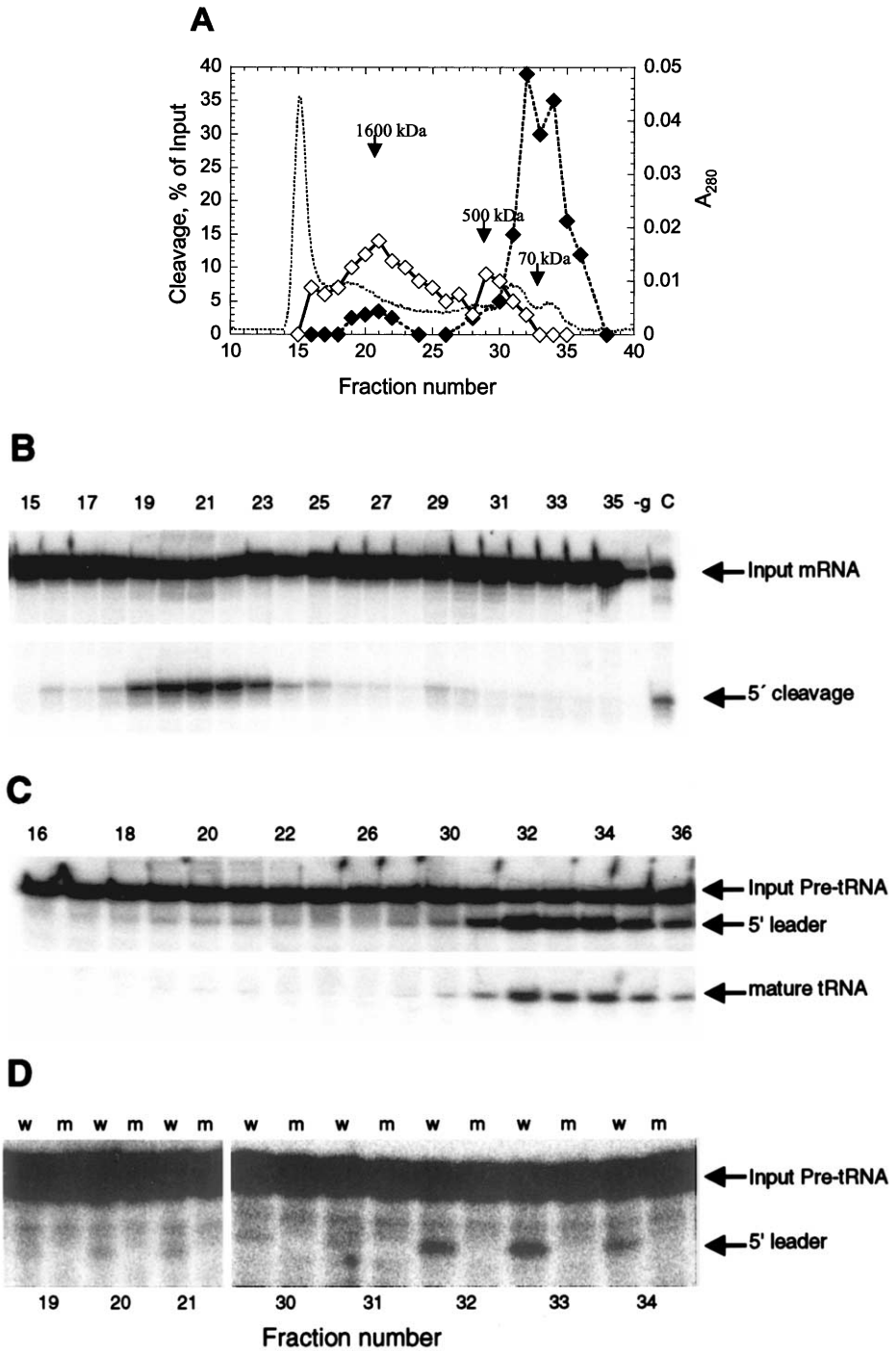

Fig. 2. Superose 6 fractionation of mitochondrial endoribonuclease activities. Superose 6 fractionation of pooled fractions 10–20 from Fig. 1B. Input substrates, cleavage products and fraction numbers are indicated. (A) Quantification of gRNA-dependent endonucleolytic cleavage of CYb mRNA (open diamonds) and of *S. pombe* pre-tRNA^{ser} at site cleaved by RNase P (filled diamonds). A_{280} is indicated by dotted lines. The peak of activities were compared with a separate run containing size standards. (B) Autoradiogram of gRNA-dependent cleavage of 5′ labeled Cyb mRNA at ES1 in the presence of cognate gRNA. The (c) indicates pooled fractions 10–20 from Q Sepharose that was used as a positive control for the editing assay and (− g) indicates omission of gRNA as a negative control. (C) Autoradiogram of cleavage of internally labeled *S. pombe* pre-tRNA^{ser}. (D) Autoradiogram of cleavage of wild type (w) and mutant (m) *S. pombe* pre-tRNA^{ser}. See Section 2 for details.

fraction eluted with a peak at 500 kDa (Fig. 2A and B). The second peak coincides with a peak that contains some but not all editing proteins and activities [21]. Most RNase P activity fractionated at ~70 kDa on Superose 6 gel filtration columns but a small portion co-fractionated at 1600 kDa along with the editing complex (Fig. 2A and C). The specificity of the RNase P is evident from its cleavage of wild type pre-tRNA at

the site leading to mature tRNA and the lack of this cleavage using a pre-tRNA that is mutated to disrupt the structure recognized by RNase P (Fig. 2D, [18]).

The biochemical properties of the three endonucleases were compared (Fig. 3). All three activities were eliminated by heating to temperatures as low as 45 °C and by digestion with proteinase K, indicating that proteins are essential to all three activities. The optimum temperature for all three activities was 28 °C, the same as the optimum growth temperature for procyclic *T. brucei*. The effect of pH on the activities varied with the buffer used. The optimum pH for the editing-associated endonuclease and RNase P activities is about 7.5 with MOPS buffer, but is about 7.0 with 50 mM Tris–HCl while it is about 7.5 for the gRNA-independent endonuclease. The editing-associated and the gRNA-independent endonucleases had optimum activity at about 10 mM $MgCl_2$ while that of RNase P was about 15 mM with a broader profile. The RNase P, the editing-associated, and gRNA-independent endonucleases had KCl optima at 80, 100, and 130 mM, respectively. These results reveal distinctive biochemical characteristics beyond their substrate and cleavage specificities. Therefore, the peak of endonuclease activity with deletion editing is not likely due to RNase P activity.

To determine whether the partially purified complex contains RNA, the pooled fractions 19–24 from the Superose 6 column which contained most of the in vitro editing activity were subjected to RNA isolation and hybridized with probes for pre-edited mRNA, edited mRNAs, gRNA, and tRNA (Fig. 4, [25]). Abundant pre-edited A6 RNA, some A6 edited, and gA6[14]gRNA were detected but tRNA was not detected. All four species were also detected in RNA extracted from total mitochondrial extracts. A non-specific RNA of 110 nucleotides long generated from a pBluescript plasmid did not hybridize to any of the probes under these experimental conditions (data not shown). Thus, the fractions containing editing complexes and gRNA-dependent endonuclease activity also contain pre-edited and edited mRNAs as well as gRNAs.

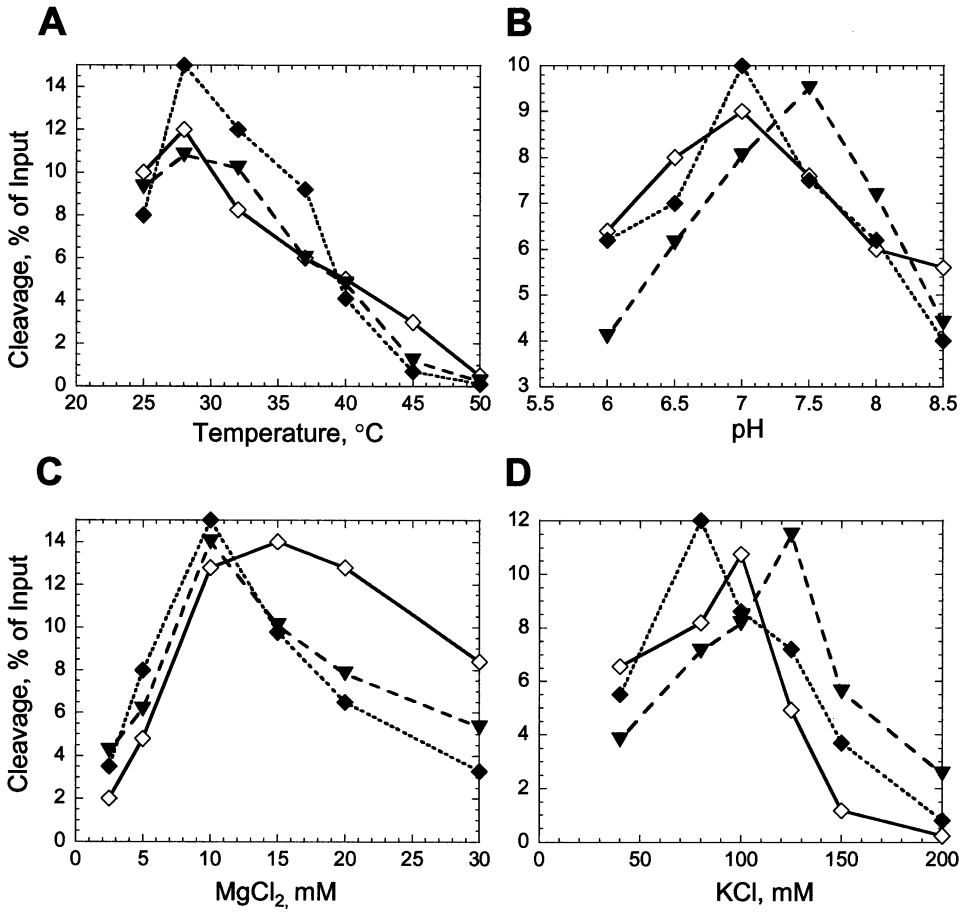

Fig. 3. Comparison of biochemical properties of *T. brucei* mitochondrial endoribonucleases. The effects of temperature, pH, $MgCl_2$, and KCl on the gRNA-dependent (open diamonds), gRNA-independent (triangles), and RNase P (filled diamonds) activities were examined as described in Section 2. The *y*-axis indicates percent of the input cleaved.

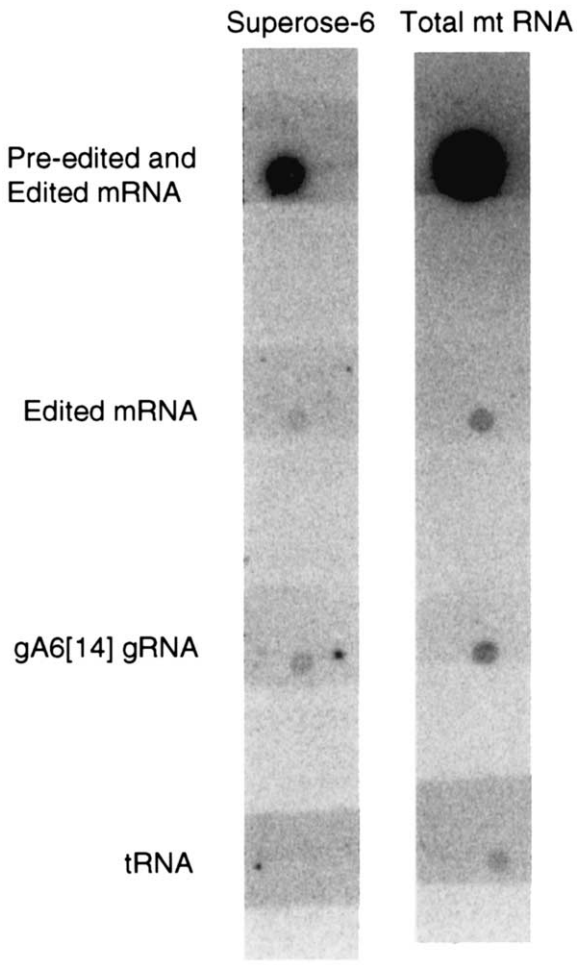

Fig. 4. RNAs present in editing fractions from Superose 6. Northern analysis of RNA extracted from pooled fractions 19–24 from a Superose 6 column or from total mitochondria. RNAs were hybridized with oligonucleotides that detect both pre-edited RNA and edited RNA, edited RNA only, gRNA, and tRNA (see Section 2 or text).

The effect of MN treatment on the editing complex was examined to assess the role of RNA in editing complex structure and activity (Fig. 5). Mitochondria were treated with 90 μg of digitonin mg^{-1} mt protein to remove the outer membrane of the mitochondrion to enhance accessibility of editing complexes to MN. Fractionation of cleared lysates of the digitonin-treated mitochondria by Superose 6 chromatography showed that endonuclease, editing and RNA ligase activities still eluted at ~1600 kDa (Fig. 5B–E). Substantial RNA also remained (Fig. 5A). These RNAs included abundant 75 nt RNA and heterogeneous 30–60 nt RNAs approximating the size of gRNAs with 3′ U-tails of variable lengths. In addition, less abundant RNAs of 80–900 nt, which may be pre-mRNA and ribosomal RNAs, were evident.

Inclusion of 800 U MN with the Digitonin treatment degraded most endogenous RNA (Fig. 5A). The 80–900 nt bands and the heterodisperse gRNA-sized RNAs were eliminated. However, the 75 nt RNA remained as did bands at approximately 32, 35, 37 nt, and a smear of small RNAs. These may represent partially degraded mRNAs, which are probably less structured than the tRNAs and gRNAs, although we cannot exclude the possibility that they represent other RNA species. Thus, the combined Digitonin and MN treatment eliminated most, but not all, of the RNAs in the pooled fraction. The total endonuclease activity remained essentially the same compared to untreated mt but precleaved editing activity was reduced by 30% (Fig. 5B–D). The gRNA-independent endonuclease co-eluted with the gRNA-dependent endonuclease from the Superose 6 column. Thus, while it is biochemically distinct from the gRNA-dependent endonuclease, this co-elution could indicate a fortuitous association or a functional association with the editing complex.

The combined Digitonin and MN treatment shifted the position of the endonuclease, in vitro editing, and RNA ligase activities by about two fractions, thus eluting at ~1100 kDa (Fig. 5B–E). The ability to detect the enzymatic activities indicates that MN was successfully inactivated by EGTA and eliminated by Superose 6 chromatography. MN treatment does not affect the RNase P activity that elutes at 70 kDa as we showed previously [18]. Moreover, RNA ligase activity fractionated into two peaks with one eluting along with the endonuclease and pre-cleaved editing activity and the other eluting at ~500 kDa, i.e. where the minor peak of editing elutes (see Fig. 2). Furthermore, the characteristics of the pre-cleaved editing are consistent with loss of ligase activity, since +2 U addition product accumulates to a higher level and ligated 0 U-addition product is reduced compared to digitonin only (Fig. 5D). This suggests that RNA degradation resulted in loss of editing complex components with some RNA ligase being lost.

4. Discussion

This study describes the fractionation of three mitochondrial endoribonuclease activities from *T. brucei*. Heat and protease sensitivity indicates that these endonucleases are proteins. Distinct combinations of temperature, pH, salt, and Mg^{2+} optima and elution profiles from successive SP-Sepharose, Q-Sepharose, and Superose 6 columns indicate that these activities are catalyzed by different proteins. One of the three endonuclease activities copurifies with in vitro editing and its cleavage is gRNA dependent. Both features are expected for an endonuclease functioning in RNA editing. A second endonuclease does not copurify with the first endoribonuclease and its activity is independent of gRNA. The third endonuclease is a mtRNase P, the

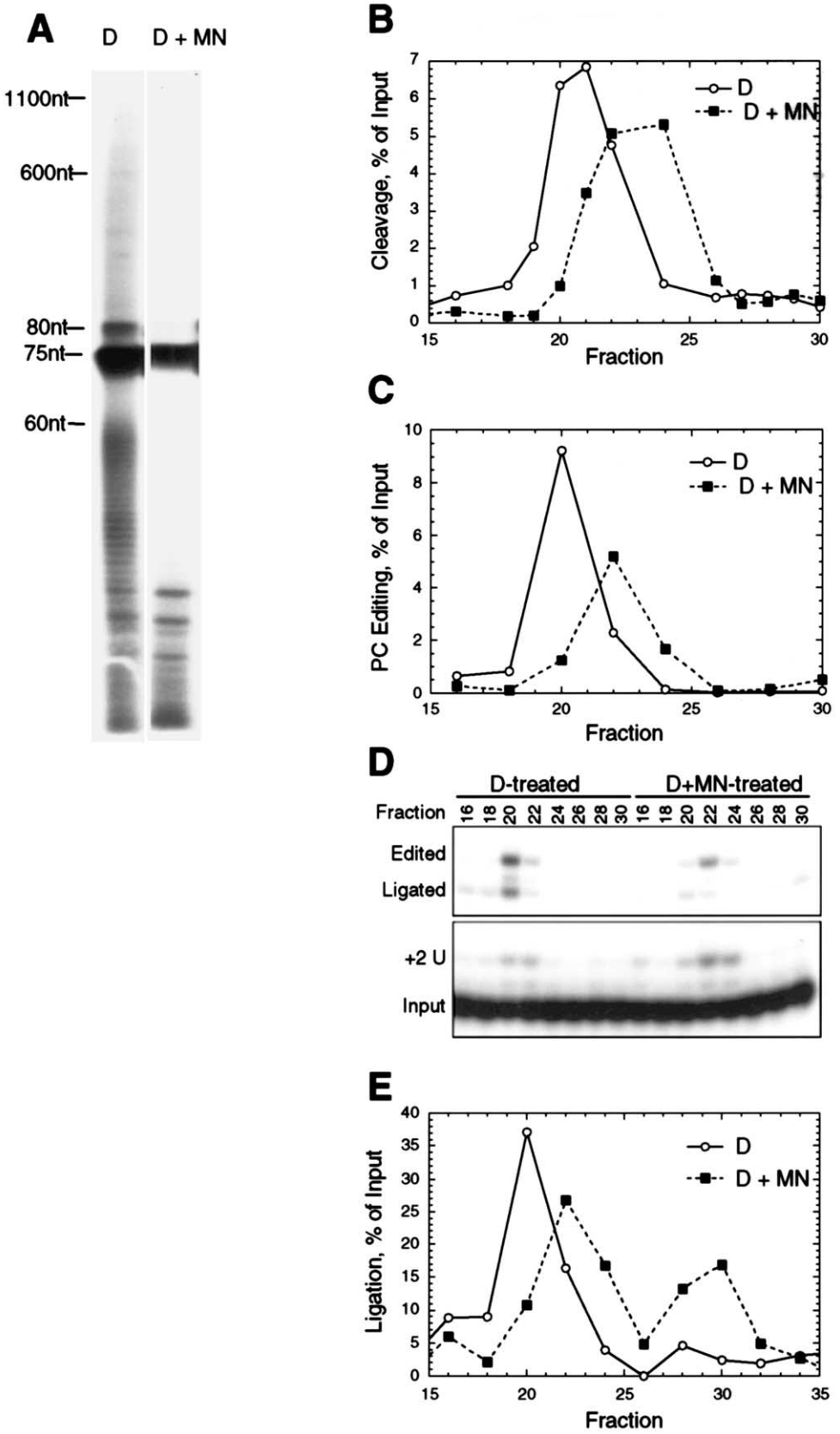

Fig. 5. Effects of digitonin and micrococcal nuclease on editing and related activities. Superose 6 fractions of mitochondria were lysed after treatment with digitonin alone (D) or with micrococcal nuclease (D + MN), as described in Section 2, and assayed for (A) RNA that can be pCp labeled, (B) gRNA-dependent enodribonuclease, (C and D) in vitro precleaved insertion editing, and (E) RNA ligase activities. All activities are expressed as percent of input RNA. Panel D shows gel used for panel C to show U-addition products. See Section 2 for details.

vast majority of which does not copurify with editing. The coelution of the gRNA-dependent and gRNA-independent endonuclease activities from Superose 6 columns suggests that both are associated with complexes although their separation on SP-Sepharose columns indicates that they are in separate complexes. The gRNA-dependent endonuclease is likely the same activity reported by others [3,26,27]. It is uncertain if this activity is catalyzed by one or more polypeptides. The editing complex contains two RNA ligases [14–16] and four related proteins, one of which may be required for editing endonuclease activity [28]. The gRNA-independent endonuclease activity may be the gRNA-independent cleavage reported by others but the activity that we observed does not require DTT, unlike that observed previously [2,10]. One possibility is that there are more than three endonucleases and the differences in enrichment protocols or other manipulations results in differential loss of some of their activities. These endonucleases may function in other RNA processing that occurs in *T. brucei* mitochondria. Such processing includes the processing of the mt rRNAs, maxicircle-encoded polycistronic pre-mRNA sequences, and gRNA precursors [29]. We cannot exclude the possibility that the gRNA-independent endonuclease activity does not have a role in editing. The in vitro editing assays only measure editing at the first or second ESs which are adjacent to the anchor duplex that results from base-pairing between the pre-mRNA and gRNA. Another endonuclease may function in cleavage events not adjacent to the anchor duplex. Such cleavages are implied by the presence of numerous partially edited junction sequences [17,30,31]. It is likely that the mt RNase P activity is required for processing of the mt tRNAs. Fractionation of this activity away from the gRNA-dependent activity in both the Q-Sepharose and Superose 6 columns and its different optima from the editing endonuclease suggests that the RNase P activity has no role in editing. The co-fractionation of a small amount of RNase P activity with the editing complex in Superose 6 columns probably indicates a fortuitous association, perhaps reflecting non-specific binding to RNA or protein. However, we cannot exclude a role in editing possibly due to sharing of a protein subunit between the two activities. Precedence exists for RNase P subunits being shared between different complexes [32].

MN treatment data, which degrades most of the RNA in the editing fraction, suggests that the editing complex contains associated RNAs. Nevertheless, not all RNA was degraded and small RNAs remain. Similarly the 80–900 nt RNA may be cleaved to the smaller size RNAs or these RNAs may resist MN degradation and thus play a role in editing. It is possible that the non-gRNA, non-pre-mRNA plays an assembly, structural, or perhaps even catalytic role in editing. The shift in Superose 6 elution resulting from MN treatment suggests that RNA is important to the structural integrity of the editing complex. All in all, these studies have provided important information on the structure of the *T. brucei* editing complex.

Acknowledgements

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