THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 10, Issue of April 5, pp. 6782-6788, 1992
Printed in U.S.A.
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

(36)

A Ribonuclease Activity Is Activated by Heparin or by Digestion with Proteinase K in Mitochondrial Extracts of *Leishmania tarentolae**

(Received for publication, November 5, 1991)

Agda M. Simpson, Norbert Bakalara‡, and Larry Simpson§

From the Department of Biology and the Molecular Biology Institute, University of California, Los Angeles, California 90024

A ribonuclease activity in a 100,000 × *g* supernatant of a Triton lysate of a mitochondrial-kinetoplast fraction from *Leishmania tarentolae* is activated by incubation with heparin or by predigestion of the lysate with proteinase k or pronase. *In vitro*-transcribed pre-edited cytochrome *b* mRNA is cleaved at several sites. With time, complete degradation of the RNA occurs. All cleavages occurred within putative single-stranded regions of the RNA. No cleavage was observed with 9 S rRNA. The presence of a nonspecific nucleotide or nucleoside slows the rate of cleavage. The cleavage activity is inhibited by sodium dodecyl sulfate or phenol/chloroform extraction, is retained by a 10-kDa cutoff filter, and passes through a 30-kDa filter. Micrococcal nuclease inhibits the proteinase-induced activity but not the heparin-induced activity.

Several ribonuclease activities have been shown to exist in mitochondria. An RNase P activity has been identified in HeLa mitochondria which cleaves the precursor to *Escherichia coli* $tRNA^{Tyr}$ at the same site as the *E. coli* RNase P, producing a mature 5′-end of the tRNA (1). It is thought that the HeLa mitochondrial RNase P is involved *in vivo* with mitochondrial tRNA processing. In mouse mitochondria, an RNA-processing endoribonuclease (RNase MRP) has been identified which cleaves mitochondrial RNA complementary to the light strand at a unique site and is thought to be involved in the initiation of mitochondrial DNA replication (2). The 136-nt[1] RNA associated with this enzyme is a nuclear gene product also found associated with the nucleolus (3–5). The mitochondrial MRP and mammalian RNase P share a common antigenic epitope (6), but it has been shown that RNase MRP and HeLa mitochondrial RNase P are different enzymes.[2] In yeast, an RNase P activity which is involved in 5′ processing of mitochondrial tRNAs has been demonstrated in purified mitochondria (7). This enzyme has an RNA component which is encoded at the tRNA synthesis locus in *Saccharomyces cerevisiae* (8) and a homologous locus in *Candida glabrata* (9). Another endoribonuclease activity has been detected in yeast mitochondria which removes the 3′ trailer sequences from 5′-matured tRNA precursors (10).

In the kinetoplast-mitochondrion of the kinetoplast protozoa, nothing is known about the ribonucleases which almost certainly are involved with the processing and turnover of mitochondrial rRNAs, mRNAs, and guide RNAs (gRNAs) transcribed from the maxicircle and minicircle DNA molecules (11). The post-transcriptional RNA modification system known as RNA editing, which inserts and deletes uridine (U) residues from mRNAs, may also require the assistance of specific endo- and exoribonucleases (12–15). Finally, the apparent tRNA importation system may require specific nucleases for RNA processing (16, 17).

In this paper, we demonstrate the presence of a cryptic RNA cleavage activity in mitochondrial extracts from *Leishmania tarentolae*, which is activated by treatment with heparin or by digestion with proteinase K, and discuss the possible role of this activity in RNA processing.

EXPERIMENTAL PROCEDURES

Cell Culture and Mitochondrial Isolation—*L. tarentolae* cells (UC strain) were grown in brain heart infusion medium (Difco) at 27 °C as described previously (18). The mitochondrial-kinetoplast fraction was isolated from late log phase cells (100–180 × 10^6 cells/ml) by flotation in Renografin density gradients after cell rupture under hypotonic conditions as described previously (18, 19). The final washed mitochondrial pellet was resuspended at 5 mg of protein/ml in 20 mM Hepes (pH 7.5), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, and stored frozen in 200-μl aliquots at −80 °C.

Preparation of Mitochondrial Extracts—The thawed mitochondrial suspension (200 μl) was homogenized for 15 s at 5 °C with 0.3% Triton X-100 (Pierce) in a Microfuge tube (Kontes, Pellet Pestle Mixer, motor-driven), yielding the TL extract (20). Centrifugation of TL for 30 min in a Microfuge at 12,000 × *g* yielded the TS extract (20). Centrifugation of TS for 100,000 × *g* for 60 min yielded the S-100 extract. Proteinase K (Bethesda Research Laboratories) was dissolved at 10 mg/ml in 50 mM Tris-HCl (pH 8), 1 mM $CaCl_2$. Predigestion of the TL or the TS extract was performed by addition of 100 μg/ml proteinase K and incubation for 5 min at 37 °C.

In Vitro Transcription—A 198-base pair *AccI/RsaI* restriction fragment from the pLt120 maxicircle region (nucleotides 5347–5545 of the *L. tarentolae* maxicircle sequence; entry LEIKPMAX in GenBank) was cloned into the *SmaI* site of the pBluescript SK (−) vector (Stratagene) to yield the pNB2 plasmid. pNB2 plasmid DNA digested with *BamHI* was used as template to synthesize RNA using T7 polymerase, as described previously (20). For uniformly labeled (UL) RNA, the concentration of cold UTP was decreased 3-fold and 10 μCi of [^{32}P]UTP (800 Ci/mmol) was added. The 272-nt pNB2 RNA (5′ to 3′) contained a 73-nt vector sequence at the 5′ end followed by 199-nt maxicircle sequence. This sequence represented the 5′ portion of the cytochrome *b* (CYb) gene, containing the 22-nt pre-edited region (PER) with a 56-nt sequence 5′ flanking and 186-nt sequence 3′ flanking. RNA was recovered from the preparative acrylamide gel by diffusion and ethanol precipitation.

The 9 S rRNA coding sequence (21) was PCR-amplified from the

* This work was supported in part by National Institutes of Health Grant AI-09102. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Molecular Parasitology, University of Bordeaux II, 146 Rue Leo Saignat, Bordeaux Aquitaine, 33000 France.

§ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: nt, nucleotide(s); gRNA, guide RNA; CYb, cytochrome *b*; TUTase, terminal uridylyltransferase; TL, Triton lysate; TS, Triton supernatant; S-100, 100,000 × *g*-1 h supernatant of TS; PER, pre-edited region; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; AMP-PNP, adenyl-5′-yl imidodiphosphate.

[2] G. Attardi, personal communication.

pLt120 cloned maxicircle DNA (nt 1639–2249 in LEIKPMAX); the 5′ PCR primer contained a T7 promoter sequence. *In vitro* transcription of the PCR product with T7 polymerase yielded the 611-nt 9 S rRNA, which was used as a control in the cleavage experiments.

Cleavage Reaction—The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, approximately 10^4 cpm of uniformly labeled (UL) RNA (10^8 cpm/μg), and 5 μg/ml heparin (Sigma). Other nucleotides or nucleosides, when substituted for ATP, were used at 1 mM. Mitochondrial extract (TL, TS, or S-100) (10 μl/50 μl reaction volume) was added to start the reaction. After a 1-h incubation at 27 °C, the reaction was terminated by extraction with phenol/chloroform, and the RNA was analyzed in an 8% acrylamide, 7 M urea gel. The gel was fixed and dried for autoradiography.

Micrococcal Nuclease and Mung Bean Nuclease Digestion—Micrococcal nuclease (Pharmacia LKB Biotechnology Inc.) stock solution was 8 units/μl in 10 mM Tris-HCl (pH 8), 1 mM $CaCl_2$. Digestion of TL or TS extract was performed by addition of 1 mM $CaCl_2$ and 16 units of micrococcal nuclease to a 50-μl extract and incubation for 30 min at 4 °C. The reaction was terminated by addition of 2.5 mM EGTA.

Limiting digestion of pNB2 RNA with 3.2×10^{-4} units of micrococcal nuclease was performed in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM $CaCl_2$, for 30 min at 4 °C. Limiting digestion of pNB2 RNA with 1.6×10^{-3} units/μl mung bean nuclease (Bethesda Research Laboratories) was performed in 30 mM sodium acetate (pH 5), 50 mM NaCl, 1 mM zinc acetate, 5% glycerol, 50 μg/ml bovine serum albumin, for 20 min at 27 °C.

Centrifugal Filtration of S-100 Mitochondrial Extract—The TL extract was predigested with proteinase K and centrifuged at 100,000 × *g* for 1 h to obtain the S-100 extract. This extract was subjected to centrifugal filtration (2000 × *g* for 40–90 min) through Millipore Ultrafree-MC 10,000 NMWL or 30,000 NMWL filters. The filtrate was assayed for cleavage activity in the presence of heparin using pNB2 RNA as substrate.

RNA Sequencing—The pNB2 RNA cleavage products were recovered from a preparative gel and used as template for dideoxy chain termination sequencing as described previously (12). The sequencing primer (5′-CCTAAACTAAACCTACACC-3′) (nt 5475-5456 in GenBank entry LEIKPMAX) was 5′-end-labeled with [γ-^{32}P]ATP (>7000 Ci/mmol; ICN) and T4 polynucleotide kinase (Bethesda Research Laboratories).

Computer Analysis—The pNB2 RNA sequence was analyzed for secondary structures by the FOLD program of Zuker and Stiegler (22).

RESULTS

Presence of Heparin Induces Ribonuclease Activity in Mitochondrial Extract—T7-synthesized pre-edited cytochrome *b* mRNA (pNB2 RNA) is not degraded by 1-h incubation at 27 °C with unclarified or clarified mitochondrial Triton extracts (TL or TS extracts). A sonicated mitochondrial extract without Triton also showed an absence of ribonuclease activity (data not shown). pNB2 RNA was, however, extensively labeled under TUTase conditions (see "Experimental Procedures") in the presence of TL extract with [^{32}P]UTP without any degradation. However, if heparin was present to inhibit the activity of the TUTase (20), UL pNB2 RNA was degraded into several specific fragments after the standard 40-min incubation at 27 °C, suggesting that a nuclease was activated which cleaved the RNA at several specific sites (Fig. 1*A*). This was surprising since heparin has not been previously reported to stimulate RNase activity.

The standard pNB2 RNA cleavage reaction consisted of the addition of 1/5 volume of TL, TS, or S-100 extract to pNB2 RNA in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP for 1 h at 27 °C. The addition of heparin (5 μg/ml) was required to activate the cleavage activity. The RNA cleavage products were analyzed in an 8% acrylamide, 7 M urea gel. The ATP requirement will be discussed below.

Predigestion of the Mitochondrial Lysate with Proteinase K or Pronase Also Induces Ribonuclease Activity—An identical pNB2 RNA cleavage pattern was obtained in the absence of heparin by a brief predigestion of the TL extract with proteinase K (Fig. 1*A*); addition of heparin during the reaction was synergistic. The effectiveness of the protease digestion in degrading protein was confirmed by SDS gel analysis (data not shown). Predigestion with another broad spectrum protease, pronase B, also induced this specific cleavage activity (Fig. 1*B*). Activation of the cleavage activity also occurred when the Microfuge-clarified extract (TS extract) was extracted with StrataClean (Stratagene) resin (data not shown). This silica-based resin contains hydroxyl groups which interact with proteins in a manner similar to the hydroxyl groups of phenol and thereby remove the proteins from solution.

However, deproteinization with phenol/chloroform or addition of 0.2% sodium dodecyl sulfate both effectively inhib-

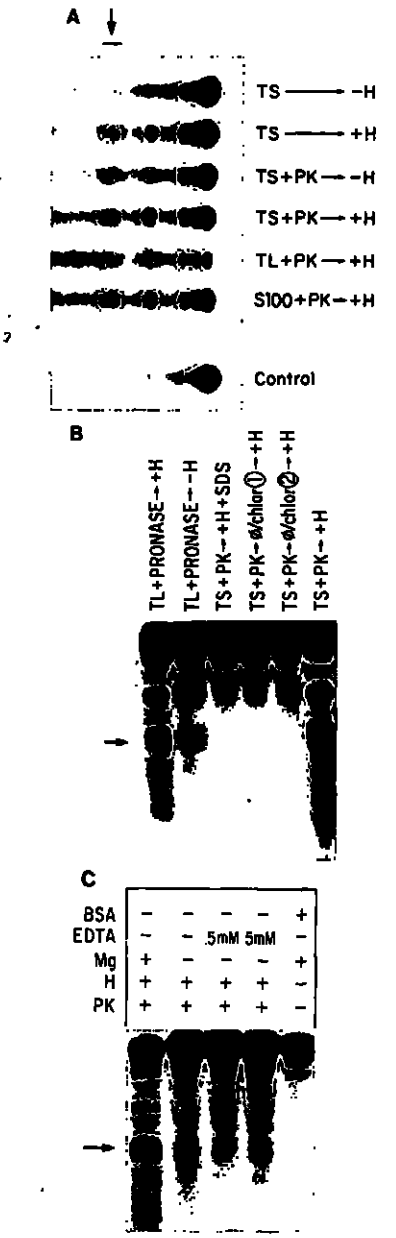

FIG. 1. **Activation of cleavage of pNB2 RNA by addition of heparin to TL or TS extract or by predigestion with proteinase K or pronase.** Uniformly labeled (UL) pNB2 RNA was incubated with TS, TL, or S-100 extract as indicated above each lane. *−H*, no heparin added; *+H*, heparin added; *PK*, proteinase K predigestion; *Control*, no incubation. The *bracket* and *arrow* indicate the two major products resulting from cleavage within the PER. *A*, activation by heparin or proteinase K digestion. *B*, effect of pronase, SDS, or phenol/chloroform treatment on cleavage activity. *C*, Mg^{2+} requirement of cleavage activity.

ited the proteinase K or heparin-activated cleavage activity, as shown in Fig. 1*B*. These data suggest that the cleavage activity is probably due to a protease-resistant protein and not to a catalytic RNA. Fig. 1 also shows that Microfuge centrifugation of the TL extract to produce the TS extract or high speed centrifugation to produce the S-100 extract have no effect on the proteinase K + heparin-inducible cleavage activity, suggesting that the induced activity is not membrane-bound. Proteinase K digestion of S-100 extract also induces the activity (data not shown), indicating that the activity is in a soluble form prior to protease digestion.

Substitution of bovine serum albumin for the proteinase K in the predigestion of the TL extract did not activate the cleavage activity (Fig. 1*C*), indicating that the activation by proteinase K was not a nonspecific phenomenon caused by protein binding of some cofactor.

The presence of Mg^{2+} was required for proteinase K + heparin-induced cleavage activity. As shown in Fig. 1*C*, deletion of $MgCl_2$ from the reaction mix and removal of endogenous Mg^{2+} by the addition of EDTA inhibited the reaction.

The proteinase K + heparin-induced activity of the TL extract did not affect pNB2 plasmid DNA in a specific manner, but a nonspecific DNase activity was observed in the TL extract, which was inhibited by the addition of heparin.

Major Cleavage Sites of pNB2 RNA Are within the Pre-edited Region—The two approximately 140-nt major cleavage products indicated by a bracket in Fig. 2*A* were gel-isolated and subjected to sequence analysis using a primer downstream of the pre-edited region. As shown in the sequencing gel of Fig. 2*B*, a strong termination in all lanes occurred between 2 G residues, as indicated by the nucleotide labeled N·. This was interpreted as being due to truncated molecules produced by a specific cleavage. Four other less frequent cleavages also occurred in this region. Also indicated in this figure on the left side are the location of sites of addition of U residues in the mature edited mRNA. It may prove relevant that the major cleavage site is located 2 nt upstream of the first expected editing site (23) and that the four minor cleavages are also located within the pre-edited region (PER).

The other cleavage sites giving rise to the three higher molecular weight bands were not precisely mapped, but from the sizes (220 nt, 190 nt, 180 nt) of the fragments and their specific 5′ end-labeling with [γ-^{32}P]ATP and T4 kinase, these sites are localized outside the PER (data not shown). The relative amounts of these three cleavage products as compared to the 140-nt products varied between experiments (compare the patterns in Figs. 1*A* and 2*A* with those in Figs. 4 and 5), but conditions can be obtained in which the 140-nt bands represent the major cleavage products.

The Proteinase K + Heparin-induced Nuclease Activity Does Not Affect the Mitochondrial 9 S rRNA—Mitochondrial 9 S rRNA is apparently not internally or 5′-edited *in vivo* (24). T7-synthesized 9 S RNA was unaffected by incubation for 1 h at 27 °C in the presence of proteinase K + heparin-treated TL extract under conditions in which pNB2 RNA is cleaved (data not shown).

Ultrafiltration of the Cleavage Activity Indicates a Molecular Size between 10 kDa and 30 kDa—As shown in Fig. 3, the proteinase K + heparin-activated pNB2 RNA cleavage activity was retained by a 10-kDa cutoff centrifugal filter (Millipore) and passed through a 30-kDa cutoff filter. This suggests that the cleavage activity consists of one or more components that have a molecular size between 10 and 30 kDa.

Presence of Nucleotide Affects Rate of Cleavage Reaction—Removal of ATP from the standard TL reaction mixture resulted in complete degradation of pNB2 RNA to small fragments after a 1-h incubation at 27 °C (Fig. 4). However, ADP, AMP or a nonhydrolyzable analogue of ATP, AMP-PNP, each could substitute for ATP in producing the characteristic cleavage pattern (Fig. 4*A*). Other nucleotides had varying levels of effectiveness in substituting for ATP. As shown in Fig. 4*B*, AMP or dATP yielded a similar cleavage pattern as ATP, CTP and UTP had little effect, and GTP almost completely inhibited the cleavage under standard reaction conditions. Adenosine was as effective as ATP (data not shown). The nucleotide effect was apparently on the

FIG. 2. **Localization of sites of cleavage within PER.** *A*, preparative gel of cleaved pNB2 RNA for sequence analysis. The *bracket* and *arrow* indicate the two bands that were gel-isolated. *REF*, *L. tarentolae* cytoplasmic rRNA and tRNA molecular weight markers. *B*, sequencing ladders of pNB2 RNA and the cleavage products from *A*. The sites and number of U residues added in editing are indicated on the *right*. The cleavage sites are indicated by *arrows* and by N· on the *right*.

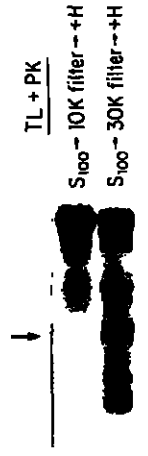

FIG. 3. **Centrifugal filtration of the cleavage activity.** TL extract was digested with PK and then clarified at 100,000 × *g* for 1 h. The resulting S-100 supernatant was subjected to centrifugal filtration through a Millipore 10-kDa-cutoff filter or a 30-kDa cutoff filter. Each filtrate was tested for cleavage activity using UL pNB2 RNA as shown.

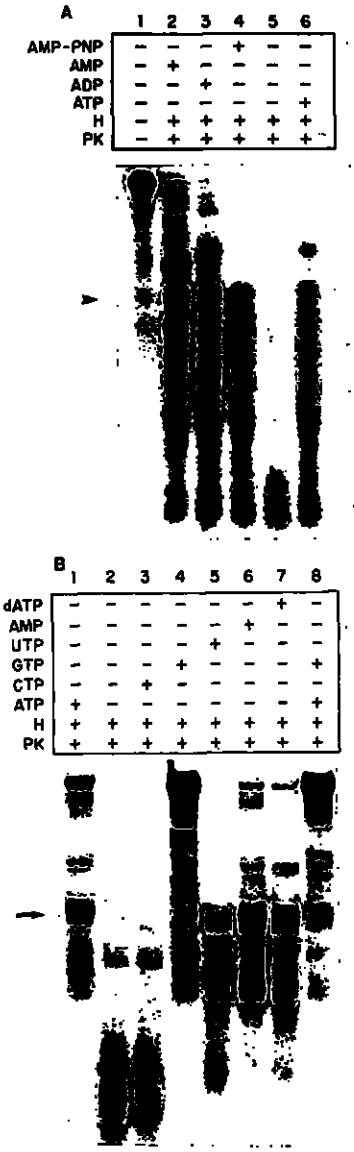

FIG. 4. **Nucleotide requirement of cleavage activity.** UL pNB2 RNA was treated with TL extract under standard cleavage conditions. *PK*, proteinase K-predigested; *H*, heparin added to cleavage reaction mixture. *A*, the presence or absence of ATP, ADP, AMP, or AMP-PNP is indicated above each lane. The *arrow* indicates the localization of the two products produced by cleavage within the PER. *B*, substitution of CTP, GTP, UTP, AMP, and dATP for ATP.

kinetics of cleavage, as GTP at 37 °C gave a similar pattern as ATP at 27 °C, whereas ATP at 37 °C produced complete degradation and at 4 °C inhibited cleavage completely (Fig. 5*A*). This was confirmed by the experiment in Fig. 5*B*, in which incubation of pNB2 RNA in TL extract at 4 °C in the absence of any added nucleotide yielded the identical cleavage pattern as with ATP at 27 °C.

The 3′ OH of the Nucleotide Cofactor Is Not Required for Inhibition of Cleavage Activity—Both cordycepin (3′-deoxy-ATP) and 2′,3′-dideoxy-ATP could substitute for ATP in the standard pNB2 RNA cleavage reaction, suggesting that the 3′ OH of the added nucleotide is not required (Fig. 6). In addition, [α-^{32}P]-ATP was not covalently linked to the cleaved fragments after the reaction (data not shown). This evidence and that presented above clearly showed that the nucleotide requirement was not due to a 3′ OH nucleophilic attack and transesterification reaction analogous to the role of guanosine or GMP in group I splicing (25).

Micrococcal Nuclease Prevents the Activation of the Cleavage

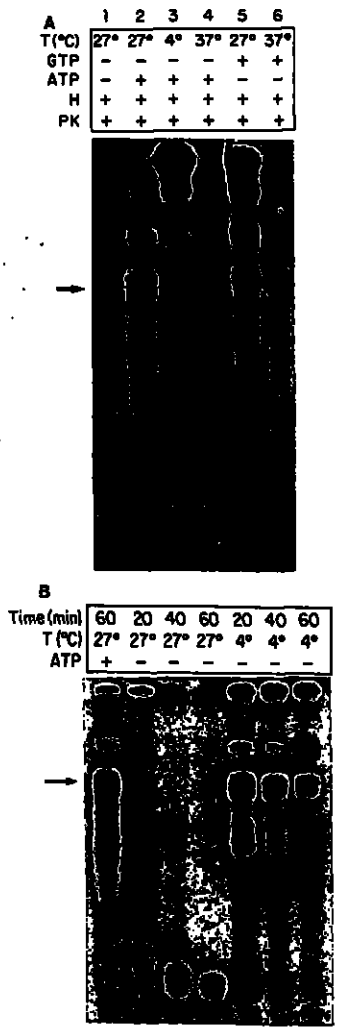

FIG. 5. **Effect of temperature of incubation on cleavage of pNB2 RNA in presence of ATP or GTP.** UL pNB2 RNA was treated in the presence of heparin and either ATP or GTP with proteinase K (*PK*)-predigested TL for the indicated times at the indicated temperatures. *A*, incubation at 37 °C with GTP produces the same result as incubation at 27 °C with ATP. *B*, cleavage reaction in absence of ATP at 4 °C resembles that in presence of ATP at 27 °C.

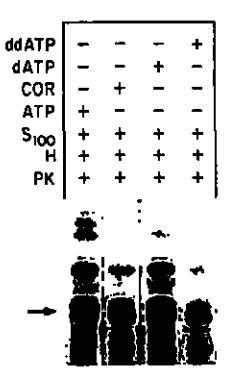

FIG. 6. **The 3′-OH is not required for the ATP effect on the cleavage reaction.** Details are shown above each lane. *COR*, cordecypin.

Activity by Proteinase K Digestion but Not by Heparin—Digestion of the proteinase K-digested TS extract with micrococcal nuclease completely inhibited the proteinase K-induced pNB2 RNA cleavage activity (Fig. 7*A*). However, the inhibitory effect of micrococcal nuclease on the proteinase K-induced cleavage activity was also observed when the micro-

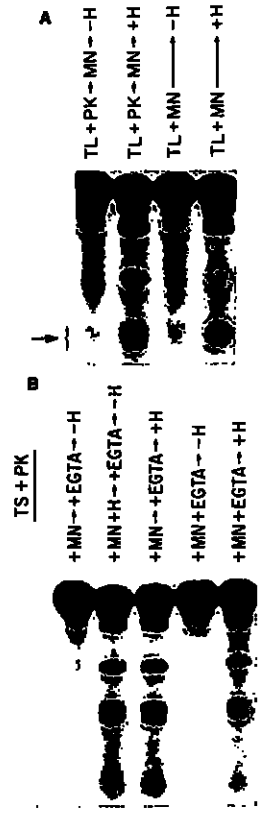

FIG. 7. **Micrococcal nuclease inhibits proteinase K-activated nuclease activity and heparin restores activity.** Details are shown above each lane. *MN*, micrococcal nuclease digestion. The micrococcal nuclease digestion was terminated by addition of 2.5 mM EGTA. *−H* or *+H*, cleavage reaction performed in absence or presence of heparin. *A*, effect of micrococcal nuclease digestion on induction of cleavage activity by proteinase K (*PK*). *B*, EGTA-inhibited micrococcal nuclease also inhibits the proteinase K-induced cleavage activity.

coccal nuclease digestion was performed in the presence of EGTA, which inhibits the nuclease activity of micrococcal nuclease by removal of Ca^{2+} (Fig. 7*B*), indicating that the micrococcal nuclease inhibition effect was not due to degradation of an RNA component.

Addition of heparin during the cleavage reaction or during the micrococcal nuclease predigestion reactivated the micrococcal nuclease-inhibited cleavage activity (Fig. 7, *A* and *B*). Gel analysis of the treated lysate showed that micrococcal nuclease digestion (in the absence of EGTA) was effective in degrading RNA, including gRNA, and that the addition of EGTA completely inhibited the digestion of RNA (data not shown). Control experiments also demonstrated that the presence of the proteinase K during the 30-min treatment with micrococcal nuclease at 5 °C did not greatly affect the nuclease activity of the micrococcal nuclease itself (data not shown).

This evidence indicates that micrococcal nuclease is inhibiting the PK-induced pNB2 RNA cleavage activity by binding a cofactor (26) rather than by degrading RNA. Heparin would then release the bound cofactor.

Limiting Digestion of pNB2 RNA with Mung Bean Nuclease and Micrococcal Nuclease—UL pNB2 RNA was digested with limiting amounts of mung bean nuclease and micrococcal nuclease, and the digestion patterns compared with that produced by digestion with the proteinase K + heparin-activated mitochondrial cleavage activity (Fig. 8). A discrete series of bands is produced by limiting micrococcal nuclease and mung bean nuclease digestion, indicating the presence of labile regions in the intact pNB2 RNA molecule. The mung bean

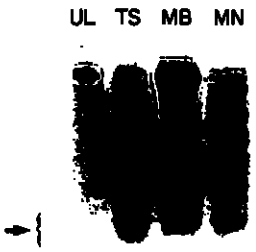

FIG. 8. **Comparison of the patterns produced by limiting digestion of pNB2 RNA with micrococcal nuclease and mung bean nuclease with the proteinase K + heparin-activated cleavage pattern.** *UL*, UL pNB2 RNA untreated control. *TS*, UL pNB2 RNA digested with proteinase K + heparin-treated TS for 1 h at 27 °C. *MB*, UL pNB2 RNA digested with limiting mung bean nuclease. *MN*, UL pNB2 RNA digested with limiting micrococcal nuclease. *Arrow* indicates two fragments produced by cleavage within PER in the TS lane.

nuclease gives rise to four bands running at approximately the same position as the upper four bands obtained with proteinase K + heparin cleavage. Three of the micrococcal nuclease bands also co-migrate with proteinase K + heparin bands. However, the two prominent cleavage fragments in the proteinase K + heparin-digested RNA which are produced by a major cleavage just upstream of editing site 1 correspond in the micrococcal nuclease or mung bean-digested RNA to a single band, suggesting that the sites of cleavage are not identical.

DISCUSSION

This investigation was stimulated by the observation that synthetic labeled pre-edited CYb RNA (pNB2 RNA) is not degraded by incubation for 1 h at 27 °C in the presence of the mitochondrial TL extract under conditions in which the mitochondrial TUTase (20) adds multiple 3′ terminal U residues. However, if the TUTase activity is inhibited by the addition of 5 μg/ml heparin, the exogenous pNB2 RNA is cleaved into several specific fragments during this incubation. Heparin is apparently activating a latent ribonuclease which has some specificity. The fact that the identical cleavage pattern occurred if the TL extract was preincubated with a broad spectrum protease such as proteinase K or pronase suggests that the same ribonuclease activity can be activated in several ways and may be due to the same enzyme. However, it is possible that different ribonucleases are activated by proteinase K digestion or by heparin which have the same substrate specificity. The pNB2 cleavages all occur within putative single-stranded regions, as determined by the FOLD program (Fig. 9). A major early cleavage site was precisely mapped by sequencing cleaved fragments to be 2 nt upstream from the first expected site of uridine addition in the mature edited RNA and a cluster of four other cleavages were all within the pre-edited region. With longer incubation periods, several additional cleavages occurred at other sites in the pNB2 RNA, eventually producing complete degradation.

The nonspecific requirement of a nucleotide for the accumulation of the major cleaved fragments at room temperature appears to be due to an inhibitory effect on the reaction which slows the kinetics of the fragmentation reaction at 27 °C and allows accumulation of the two specific fragments resulting from cleavage within the PER. Continued incubation results in multiple additional cleavages and complete fragmentation of the RNA. The mechanism of this inhibition is not understood, but it does not involve hydrolysis of the nucleotide nor require a 3′-OH group. We suggest that the nucleotide effect may be indirect due to the fact that this is a crude extract.

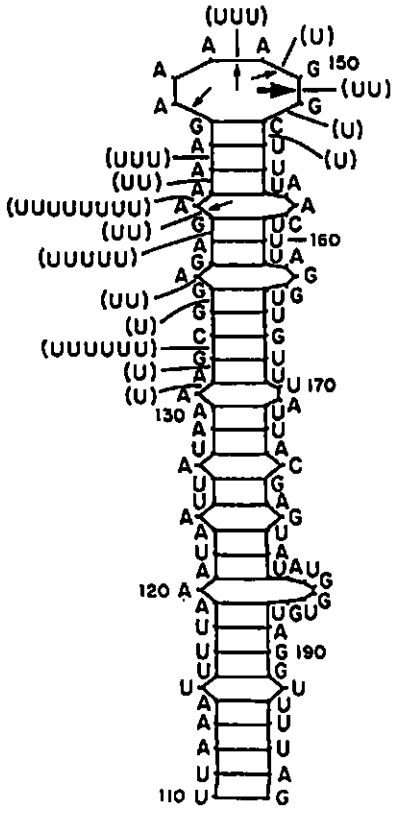

FIG. 9. **Secondary structure of pNB2 RNA predicted by FOLD program.** The number and location of U residues added by editing in the mature edited mRNA are indicated. The *dark arrow* indicates the major cleavage site and the *light arrows* the minor cleavage sites within the PER.

The molecular nature of the proteinase K + heparin-activated ribonuclease is still obscure. The resistance to broad spectrum proteolytic enzymes would appear to suggest the presence of a catalytic RNA component, similar to the known mitochondrial RNase P (1, 8) or MRP (6) enzymes. However, the sensitivity to SDS and to deproteinization by other means and the resistance of the heparin-activated activity to micrococcal nuclease digestion suggest a protein-based activity. The apparent single strand specificity of the heparin-activated activity suggests a possible relationship to a nonspecific single strand ribonuclease. The presence of nuclease-sensitive regions in the native pNB2 RNA molecule was confirmed by limiting digestions with mung bean nuclease and micrococcal nuclease. However, the fact that the fragment patterns and stoichiometries produced by these two activities are not identical indicates that the mitochondrial cleavage activity may possess additional specificities for recognition of cleavage sites other than the recognition of single-stranded regions.

The function of the proteinase K + heparin-activated ribonuclease in mitochondrial RNA metabolism is not known. RNA turnover has been shown to represent an important control mechanism controlling the steady state levels of the 9 S and 12 S rRNAs during the African trypanosome life cycle (27). One possible function of the kinetoplast ribonuclease is that this activity represents an RNA degradation enzyme involved *in vivo* with RNA turnover, which has some specificity for single-stranded regions. However, it is hard to understand why this activity would be activated by such divergent methods as heparin incubation or protease digestion.

RNA processing represents another possible role of the mitochondrial ribonuclease. There is some evidence for the existence of high molecular weight precursors for maxicircle transcripts which must be processed to yield the mature species (28–30).

Another possible function is in the process of RNA editing of maxicircle mRNAs. Two models for editing have been proposed (13, 14), and, in both models, a cleavage activity is invoked. However, to explain the activation of the cleavage activity by heparin or proteinase K digestion in the case of the enzyme cascade model, one has to propose that heparin inhibition of the internal addition of U residues, which is postulated to occur in this model by the TUTase-mediated addition of Us to the 3′-OH, prevents proper religation, thereby causing an increase in the steady state abundance of cleaved molecules. Destruction of the TUTase and the RNA ligase (20) by digestion with proteinase K would also prevent religation from occurring in this model and cause an apparent activation of the cleavage activity.

The micrococcal nuclease digestion evidence for the lack of a requirement for an RNA cofactor for the observed *in vitro* cleavage would appear to contradict the hypothesis of the cascade model that hybridization of pre-edited mRNA with a specific gRNA determines the sites of cleavage. However, the secondary structure of the pre-edited pNB2 CYb mRNA, as shown in the computer-generated "fold" in Fig. 9, emulates the gRNA/mRNA hybrid and presents mismatched bases adjacent to a duplex region. Consistent with this hypothesis is the fact that the 220-nt, 190-nt, and 180-nt minor pNB2 RNA cleavage fragments appear to be derived from cuts at single-stranded regions in this stem-loop structure. We speculate that, *in vivo*, the action of the cleavage enzyme would be limited to the PER in the mRNA/gRNA hybrid and not digest other single-stranded regions due to binding of this factor to an editing complex[3] which undergoes a specific interaction with the PER. This *in vivo* binding has not been examined.

The proteinase K + heparin activation effect can be better explained in terms of the transesterification model for editing (14). It has been shown that, in the case of group I splicing, hydrolysis at normal sites of transesterification occurs in the absence of the guanosine nucleoside which provides the attacking 3′-OH group (25). We propose in the case of our *in vitro* reaction that the observed cleavages of pNB2 RNA represent hydrolysis catalyzed by a protease-resistant catalytic core in the absence of activated gRNA. We propose that the 10–30 kDa "cleavage activity" may actually represent the catalytic core or a portion of the catalytic core of an enzyme involved normally with gRNA/mRNA transesterification. We have shown recently[3] that gRNA in the mitochondrion is not free but is bound to a 13 S TUTase complex of 6–8 proteins. Treatment with heparin produces a decrease in the sedimentation coefficient to 8 S possibly caused by the loss of several proteins from the complex. Proteinase K digestion of the complex releases the bound gRNA. We suggest that only 13 S complex-bound gRNA is active for interacting with the catalytic core enzyme and undergoing 3′-terminal transesterification with mRNA. The heparin treatment or PK digestion of the complex would then lead to hydrolysis at specific mRNA sites catalyzed by the catalytic core. Since hybridization of the mRNA with gRNA is also involved in this model in determining the sites of transesterification *in vivo*, we propose that the observed specificity of hydrolysis sites in the synthetic CYb mRNA in the *in vitro* cleavage experiments is determined by mRNA secondary structure alone and perhaps is limited to single-stranded regions, as discussed above (Fig. 9).

However, micrococcal nuclease digestion of the TL extract

[3] A. Bakker, A. Simpson, and L. Simpson, unpublished results.

in the absence of EGTA does degrade most detectable RNA, but this did not lead to activation of the pNB2 cleavage activity, as would be predicted by the above model. This result could be explained if the bound gRNA active in transesterification is protected from micrococcal nuclease digestion and if this gRNA represents a subset of total gRNA in the extract.

In conclusion, we have shown that a ribonuclease activity can be activated in a kinetoplast-mitochondrial extract by treatment with heparin or by digestion with a protease. We have discussed several possible roles of this activity. More work must be performed to establish the precise role of this activity in mitochondrial RNA processing.

Acknowledgments—We thank members of the Simpson laboratory for helpful discussions and suggestions, especially Dr. D. Maslov and Dr. B. Blum.

REFERENCES

1. Doersen, C.-J., Guerrier-Takada, C., Altman, S., and Attardi, G. (1985) *J. Biol. Chem.* **260,** 5942–5949
2. Chang, D., and Clayton, D. (1987) *EMBO J.* **6,** 409–418
3. Chang, D., and Clayton, D. (1987) *Science* **235,** 1178–1184
4. Chang, D. D., and Clayton, D. A. (1989) *Cell* **56,** 131–139
5. Chang, D. P., and Clayton, D. A. (1987) *Science* **235,** 1178–1184
6. Gold, H., Topper, J., Clayton, D., and Craft, J. (1989) *Science* **245,** 1377–1380
7. Hollingsworth, M., and Martin, N. (1986) *Mol. Cell Biol.* **6,** 1058–1064
8. Morales, M. J., Wise, C. A., Hollingsworth, M. J., and Martin, N. C. (1991) *Nucleic Acids Res.* **17,** 6865–6881
9. Shu, H.-H., Wise, C. A., Clark-Walker, G. D., and Martin, N. C. (1991) *Mol. Cell Biol.* **11,** 1662–1667
10. Chen, J. Y., and Martin, N. C. (1988) *J. Biol. Chem.* **263,** 13677–13682
11. Simpson, L. (1987) *Annu. Rev. Microbiol.* **41,** 363–382
12. Simpson, L., and Shaw, J. (1989) *Cell* **57,** 355–366
13. Blum, B., Bakalara, N., and Simpson, L. (1990) *Cell* **60,** 189–198
14. Blum, B., Sturm, N. R., Simpson, A. M., and Simpson, L. (1991) *Cell* **65,** 543–550
15. Cech, T. R. (1991) *Cell* **64,** 667–669
16. Simpson, A. M., Suyama, Y., Dewes, H., Campbell, D., and Simpson, L. (1989) *Nucleic Acids Res.* **17,** 5427–5445
17. Hancock, K., and Hajduk, S. L. (1990) *J. Biol. Chem.* **265,** 19208–19215
18. Simpson, L., and Braly, P. (1970) *J. Protozool.* **17,** 511–517
19. Braly, P., Simpson, L., and Kretzer, F. (1974) *J. Protozool.* **21,** 782–790
20. Bakalara, N., Simpson, A. M., and Simpson, L. (1989) *J. Biol. Chem.* **264,** 18679–18686
21. de la Cruz, V., Lake, J. A., Simpson, A. M., and Simpson, L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1401–1405
22. Zuker, M., and Stiegler, P. (1981) *Nucleic Acids Res.* **9,** 133–147
23. Feagin, J. E., Shaw, J. M., Simpson, L., and Stuart, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 539–543
24. Shaw, J., Feagin, J. E., Stuart, K., and Simpson, L. (1988) *Cell* **53,** 401–411
25. Cech, T. (1987) *Science* **236,** 1532–1539
26. Wang, M. J., and Gegenheimer, P. (1990) *Nucleic Acids Res.* **18,** 6625–6631
27. Michelotti, E., and Hajduk, S. (1987) *J. Biol. Chem.* **262,** 927–932
28. Feagin, J., Jasmer, D., and Stuart, K. (1986) *Mol. Biochem. Parasitol.* **20,** 207–214
29. Feagin, J., and Stuart, K. (1988) *Mol. Cell Biol.* **8,** 1259–1265
30. Simpson, A., Neckelmann, N., de la Cruz, V., Muhich, M., and Simpson, L. (1985) *Nucleic Acids Res.* **13,** 5977–5993