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Saccharomyces cerevisiae Contains an RNase MRP That Cleaves at a Conserved Mitochondrial RNA Sequence Implicated in Replication Priming

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Yeast mitochondrial DNA contains multiple promoters that sponsor different levels of transcription. Several promoters are individually located immediately adjacent to presumed origins of replication and have been suggested to play a role in priming of DNA replication. Although yeast mitochondrial DNA replication origins have not been extensively characterized at the primary sequence level, a common feature of these putative origins is the occurrence of a short guanosine-rich region in the priming strand downstream of the transcriptional start site. This situation is reminiscent of vertebrate mitochondrial DNA origins and raises the possibility of common features of origin function. In the case of human and mouse cells, there exists an RNA processing activity with the capacity to cleave at a guanosine-rich mitochondrial RNA sequence at an origin; we therefore sought the existence of a yeast endoribonuclease that had such a specificity. Whole cell and mitochondrial extracts of *Saccharomyces cerevisiae* contain an RNase that cleaves yeast mitochondrial RNA in a site-specific manner similar to that of the human and mouse RNA processing activity RNase MRP. The exact location of cleavage within yeast mitochondrial RNA corresponds to a mapped site of transition from RNA to DNA synthesis. The yeast activity also cleaved mammalian mitochondrial RNA in a fashion similar to that of the mammalian RNase MRPs. The yeast endonuclease is a ribonucleoprotein, as judged by its sensitivity to nucleases and proteinase, and it was present in yeast strains lacking mitochondrial DNA, which demonstrated that all components required for in vitro cleavage are encoded by nuclear genes. We conclude that this RNase is the yeast RNase MRP.

The overall mode of mitochondrial DNA (mtDNA) replication is best understood for the mouse and human systems, and a general model of vertebrate mtDNA replication has been established (9). Mammalian mtDNA replicates by unidirectional synthesis from two distinct origins, the origin of heavy-strand replication and the origin of light-strand replication, which are located two-thirds of the genomic distance apart on the closed circle. The structural features and mechanisms of RNA priming are very different at these two origins (3, 7, 9, 29, 30). A characteristic hallmark of the origin of heavy-strand replication is the presence of three evolutionarily conserved sequence blocks (CSBs I, II, and III) at or near the 5′ ends of newly synthesized heavy strands. It has been demonstrated for nearly all 5′ ends of nascent heavy-strand DNAs that there are RNA species whose 3′ ends map immediately adjacent to the DNA 5′ ends. It has also been shown that 5′ ends of these RNAs map at the initiation site of transcription from the major light-strand promoter (3, 7). On the basis of these and other findings, Chang and coworkers hypothesized that transcripts from this promoter play a role in heavy-strand replication by serving as primers for DNA synthesis and that individual primer termini were generated by processing of a primary transcript (3, 7).

Mammalian cells contain a site-specific ribonucleoprotein endoribonuclease (RNase MRP [mitochondrial RNA processing]) that cleaves an RNA sequence in vitro that is complementary to the origin of leading heavy-strand mtDNA replication (4, 6, 28). The endonuclease activity is present in both nuclear and mitochondrial fractions (2, 5, 14, 17, 28) and has been proposed to participate in mitochondrial primer RNA metabolism in vivo. The standard mitochondrial RNA (mtRNA) substrate for mouse and human RNase MRPs contains three short regions of sequence that are highly conserved in vertebrate species (CSBs I, II, and III). Initial characterization of the mouse RNase MRP activity demonstrated that the site-specific cleavage of mtRNA substrate in vitro occurred immediately adjacent to CSB II, and it was postulated that CSB II played a role in cleavage site specificity (4). Subsequent deletional analysis and saturation mutagenesis have determined basic substrate requirements for cleavage by mouse RNase MRP; CSB II and CSB III are essential for both efficient and accurate cleavage, whereas CSB I is not (2).

In contrast to the mammalian system, our knowledge of yeast wild-type mtDNA replication initiation, at the molecular level, is less advanced. However, studies with hypersuppressive petite strains indicate that putative yeast mitochondrial replication origins (*ori* [or *rep* {reference 12 and references therein}] sequences) are characterized by a 300-bp A+T-rich segment containing the following regions: a 16-bp A+T-rich sequence containing an active promoter for transcription initiation (termed r), a 17-bp GC cluster C located immediately downstream of the promoter, a central 200-bp A+T-rich stretch (termed ℓ), and GC clusters A and B, which are separated by an A+T-rich region (1, 10, 13). It has also been proposed that the active *ori* sequences of yeast mtDNA are origins of RNA-primed bidirectional replication (1). The available data regarding potential yeast mtDNA

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replication origins suggest possible functional relationships between the yeast and vertebrate mitochondrial systems (1, 10, 26). These include the existence of a functional promoter at or near the site of transition from RNA to DNA synthesis; the presence of a short, conserved G-rich sequence block at the origin (GC cluster C/CSB II [10, 13]); and the occurrence of an additional conserved element, CSB I, in this region (10, 13). However, we note that in some cases of severely deleted yeast mitochondrial genomes ([*rho*$^-$]), mtDNA sequence comprised solely of A · T base pairs is propagated in vivo (12).

The conserved presence of these sequence elements raised the possibility of a similar requirement for *trans*-acting factors that recognize them. In that light, we sought an endoribonuclease activity from yeast cells that might cleave a yeast mtRNA sequence containing a putative replication origin, in particular the CSB II-like region. We report here that the yeast *Saccharomyces cerevisiae* contains an endonuclease that cleaves RNA in a site-specific manner reminiscent of RNase MRP from mammalian cells.

MATERIALS AND METHODS

Reagents. Restriction enzymes, SP6 RNA polymerase, T4 DNA ligase, and RNase T_1 were from Bethesda Research Laboratories, Inc. Labeled triphosphates and [^{32}P]CDP were from New England Nuclear. T4 RNA ligase, DNase I, RNAguard, RNase T_1, RNase U2, RNase PhyM, nucleoside triphosphates (NTPs), DEAE-Sephacel, and heparin-Sepharose were purchased from Pharmacia, Inc.; micrococcal nuclease and proteinase K were from Boehringer Mannheim Biochemicals. Zymolyase was obtained from ICN Biomedicals, Inc.

Recombinant plasmids. Plasmid p64/HS40 *ori5* contains the *ori5* sequence of strain HS40 cloned into pSP64. Strain HS40, a hypersuppressive cytoplasmic petite strain with an approximately 750-bp mtDNA repeat fragment containing an *ori5* sequence (23), was obtained from R. A. Butow's laboratory. The mtDNA was digested with *Dra*I, which linearizes the repeat unit, and cloned into the *Hin*cII site of mp13. Subsequently, the mp13 clone was digested with *Hin*dIII and *Bam*HI and recloned into pSP64. Plasmid p64/HS40 *ori5*/4.4 is a 3′-deletion derivative of the aforementioned plasmid (~145 bp of yeast mtDNA). The orientations of these plasmids are such that the non-r strand (containing purines in GC cluster C/CSB II [1]) are transcribed from the SP6 promoter in these vectors. Plasmid pK408 contains the origin of heavy-strand replication of human mtDNA cloned into pSP64 (4). In these cases, the substrate transcripts produced are of the same sense as those that could prime DNA replication in vivo.

Preparation of RNA substrates. RNA substrates for in vitro cleavage were prepared by using either *Ava*II-digested p64/HS40 *ori5* (yeast substrate), *Eco*RI-digested p64/HS40 *ori5*/4.4 (yeast deletion substrate), or *Fok*I-digested pK408 (human substrate). In vitro transcriptions were carried out in a 50- or 100-μl reaction volume essentially as described by Promega (24). The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 4 mM spermidine, 10 mM dithiothreitol (DTT), 250 to 500 μM each of the four rNTPs (ATP, GTP, CTP, and UTP), 0.5 Units of RNAguard per μl, and 0.25 U of SP6 polymerase per μl. The reaction mixture was incubated at 37°C for 1 to 1.5 h at 37°C and passed over a Sephadex G-50 spin column to remove unincorporated rNTPs, and the RNA was precipitated with ethanol. RNA was 3′ end labeled in a 20-μl reaction mixture containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 15 mM $MgCl_2$, 3 mM DTT, 0.2 μg of bovine serum albumin (BSA), 10% (vol/vol) dimethyl sulfoxide, 30 μM ATP, 100 μCi of [^{32}P]pCp, and 10 U of T4 RNA ligase at 4°C for 10 to 14 h (11). The 3′-end-labeled RNA was passed over a Sephadex G-50 spin column, precipitated with ethanol, and electrophoresed in a 6% acrylamide–8 M urea gel. The RNA was excised from the gel and eluted from the gel slices by crushing and soaking in 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate–1 mM EDTA–32 U of RNAguard at 37°C for 6 to 10 h. The eluted RNA was phenol extracted three times and recovered by ethanol precipitation.

In vitro RNA cleavage assay. The cleavage assay was carried out in a 25-μl reaction mixture containing 20 mM Tris-HCl (pH 8.0) or 20 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 50 μg of BSA per ml, 4 to 16 U of RNAguard, and 4,000 to 5,000 cpm of substrate RNA at 37°C for 30 min. The reaction was terminated by adding 50 μl of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA, phenol extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol in the presence of 5 μg of *Escherichia coli* tRNA. The reaction products were analyzed by electrophoresis in 6% acrylamide–8 M urea gels.

Strains and growth conditions. *S. cerevisiae* strains used in this study were wild-type strains MH41-7B (**a** *ade his* [*rho*$^+$]), S288C (obtained from the American Type Culture Collection stock center), and BJ2168 (**a** *pep4-3 trp1*) (obtained from T. Lisowsky); [*rho*^{0}] tester strain D243-4A 18 (**a** *ade1 lys2*, [*rho*^{0}]) (obtained from T. Lisowsky); and a strain containing a disrupted mtRNA polymerase gene (*rpo41*::Tn:*URA3*), whose construction has been described in detail elsewhere (15). Yeast cells were grown to mid- to late logarithmic phase at 30°C in a medium containing 2% Bacto Peptone, 1% yeast extract, and an appropriate carbon source, either 2% glucose (YPD) or 2% galactose (YPGAL). *E. coli* HB101 and DH5 were used for cloning and were grown at 37°C in L broth.

Preparation of mitochondrial extracts. Yeast mitochondria were purified according to Christianson (8), with minor modifications. Yeast cells were harvested by centrifugation, weighed, and washed with cold distilled H_2O. The cells were resuspended in a zymolyase preincubation mix (0.02 M EDTA, 0.1 M Tris-HCl [pH 9.3], 0.1 M mercaptoethanolamine) (3 volumes per g of yeast cells) and incubated at 28 to 30°C for 30 min. Yeast cells were collected by centrifugation, washed with 3 volumes of cold 1.2 M sorbitol–1 mM EDTA, and resuspended in 3 volumes of zymolyase digestion buffer (0.2 M sodium phosphate [pH 7.4], 2 mM EDTA, 1.4 M sorbitol, 0.15 M mercaptoethanolamine). Zymolyase (150 μg/ml [60T] or 100 μg/ml [100T]) was added to the suspension and gently agitated at 28 to 30°C. The optical density at 640 nm was monitored, and when it declined to about one-fifth the original value, the spheroplasts were pelleted by centrifugation. The spheroplasts were washed twice with 3 volumes of cold 1.2 M sorbitol–1 mM EDTA and resuspended in cold breaking buffer (0.7 M sorbitol, 1 mM EDTA, 0.1% BSA). After standing on ice for 20 min, the spheroplasts were broken in 250-ml aliquots at top speed in a Waring blender for two 30- to 45-s bursts with a 2-min cooling period in between. The broken cells were centrifuged at 4,000 rpm for 10 min in a Beckman JA-14 rotor. Any remaining intact cells were resuspended in breaking buffer, broken in the Waring blender again, and then centrifuged. The supernatants were pooled and recentrifuged at 4,000

rpm for 10 min. Mitochondria were pelleted by centrifugation at 18,000 rpm for 20 min in a Beckman JA-20 rotor. The mitochondrial pellets were resuspended in breaking buffer, and the low-speed/high-speed spin cycle was repeated. Mitochondria were resuspended in breaking buffer, and the high-speed spin step was repeated two more times. The mitochondrial pellets were frozen in liquid nitrogen and stored at −80°C. In some cases, mitochondria were further purified by sorbitol or sucrose density gradient centrifugation (18). To prepare crude extracts, mitochondrial pellets were resuspended in 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–1 mM DTT, 10% glycerol (buffer A) containing 1 mM phenylmethylsulfonyl fluoride. Triton X-100 and KCl were added to 1% (vol/vol) and 0.2 M, respectively. The suspension was vigorously vortexed for 30 s every 5 min for 20 min. The crude lysates were centrifuged for 1 h at 45,000 rpm (Beckman 75Ti rotor) to remove insoluble material. Alternatively, the resuspended mitochondria were lysed by 10 strokes in a glass Dounce homogenizer and then centrifuged as described above. The crude extracts were dialyzed for 2 h against 2 liters (two changes) of buffer A plus 0.05 M KCl. Extracts were frozen at −80°C if they were not used immediately.

Preparation of whole cell extracts. One method used for the preparation of whole cell extracts was that of Lin et al. (20), with minor modifications. The procedure described is for 1 liter of cells, but it has been successfully scaled up to 8 liters. Yeast strains were grown at 30°C in YPGal to A_{600} = 4.0. The cells were harvested by centrifugation at 4,000 rpm for 5 min (Beckman JA-10 rotor) and washed with sterile cold water. The cells were resuspended in 15 ml of 1 M sorbitol–50 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–30 mM DTT and incubated for 15 min at 30°C. The cells were collected by centrifugation at 4,000 rpm for 5 min (Beckman JA-14 rotor) and resuspended in 15 ml of 1 M sorbitol–50 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–3 mM DTT. Ninety microliters of 20-mg/ml zymolyase 100T was added, and the cells were incubated with shaking at 30°C for 1 h. The spheroplasts were collected by centrifugation at 4,000 rpm for 5 min and washed two to three times with 15 ml of the digestion buffer described above. They were resuspended in 3 ml of 10 mM HEPES-KOH (pH 7.5)–1.5 mM $MgCl_2$–10 mM KCl–1 mM DTT and allowed to sit on ice for 10 to 15 min. The spheroplasts were lysed by 10 strokes in a glass Dounce homogenizer. KCl was added to a final concentration of 0.2 M, and the lysate was gently stirred on ice for 30 min. Debris was removed by centrifugation at 17,000 rpm for 30 min (Beckman JA-20 rotor). The supernatant was centrifuged at 40,000 rpm for 60 min (Beckman 70.1Ti rotor) and dialyzed for 3 h against 2 liters of 20 mM HEPES-KOH (pH 7.5) or 20 mM Tris-HCl (pH 8.0)–50 mM KCl–1 mM EDTA–1 mM DTT–20% (vol/vol) glycerol. Finally, the extract was centrifuged at 17,000 rpm for 20 min (Beckman JA-20 rotor) to remove any insoluble material, and the supernatant was frozen in liquid N_2 and stored at −80°C. Alternatively, whole cell extracts were prepared from yeast cells (2 to 6 liters) grown and harvested as described above, washed in cold distilled H_2O, and resuspended in 30 ml of 20 mM Tris-HCl (pH 8.0)–0.2 M KCl–1 mM EDTA–1 mM DTT–10% glycerol–1 mM phenylmethylsulfonyl fluoride. The cell suspension was placed in the small (50-ml) Bead Beater (Biospec) chamber, filled with 0.5-mm zirconium beads, and surrounded with an ice water jacket. The cells were disrupted with 10- to 20-s bursts, each followed by a 2-min cooling period. The suspension was centrifuged for 10 min at 5,000 rpm (Beckman JA-20 rotor) to pellet debris and any remaining beads. The supernatant was centrifuged for 1 h at 40,000 rpm (Beckman 70Ti rotor). The extract was dialyzed against 2 liters of 20 mM Tris-HCl (pH 8.0)–50 mM KCl–1 mM EDTA–1 mM DTT–10% glycerol for 2 h and frozen at −80°C.

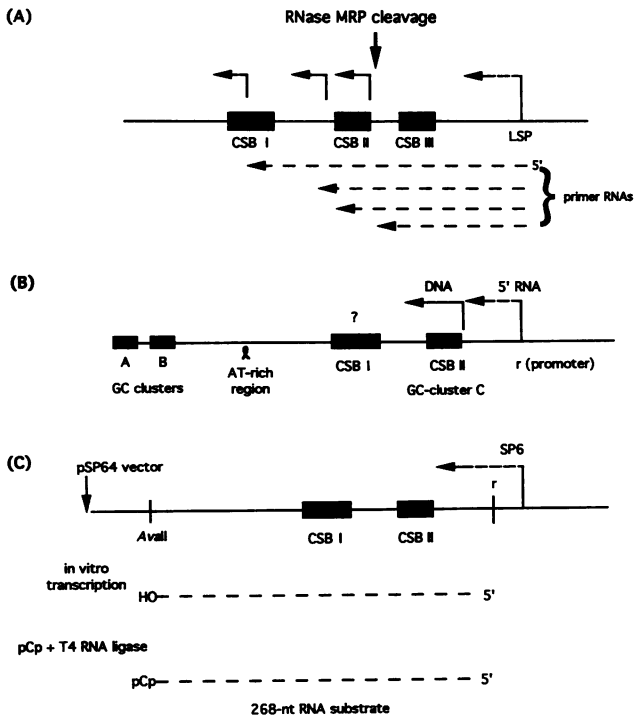

FIG. 1. Summary of the mammalian leading-strand mtDNA origin and yeast wild-type mtDNA origin region showing structural similarities. (A) The mammalian mtDNA origin. The light-strand transcriptional promoter (LSP), 5′ termini of nascent leading-strand DNAs (three short bent arrows), primer RNAs (dashed lines), and CSBs I, II, and III are shown. Also indicated is the site of cleavage by mouse or human RNase MRP immediately adjacent to CSB II (thick vertical arrow). (B) *ori/rep* region of yeast mtDNA. The structural organization of the *ori* (also termed *rep* [12]) region is shown, including the upstream promoter (r), the 5′ end of DNA (short bent arrow) mapped on the petite repeat unit by Baldacci et al. (1), the central A+T-rich region (€), and the three G+C clusters A, B, and C. GC cluster C is very similar to CSB II. The upstream location of promoters varies in distance between different *ori/rep* sequences. (C) Schematic of the 268-nt *ori5* RNA substrate used in the yeast in vitro processing reaction. SP6 RNA polymerase was used to synthesize an RNA species that was subsequently 3′ end labeled with pCp and T4 RNA ligase.

Protein purification. Yeast mitochondrial or whole cell extracts in 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–1 mM DTT–10% glycerol (buffer A) containing 50 or 100 mM KCl were applied at 20 ml/h to DEAE-Sephacel columns (10 mg of protein per g of resin) equilibrated in the same buffer. The column was washed with at least 10 column volumes of starting buffer or until the A_{280} of the effluent was <0.05. Bound proteins were eluted from the column with buffer A plus 0.3 M KCl. Fractions were tested for cleavage activity, and active fractions were pooled and dialyzed for 2 h against 2 liters of buffer A plus 0.05 M KCl. For glycerol gradient centrifugation, either crude extracts (mitochondrial or whole cell) or purified DEAE-Sephacel fractions were loaded onto 10 to 30% glycerol gradients in 20 mM Tris-HCl (pH 8.0)–50 mM KCl–0.5 mM EDTA–1 mM DTT. One hundred micro-

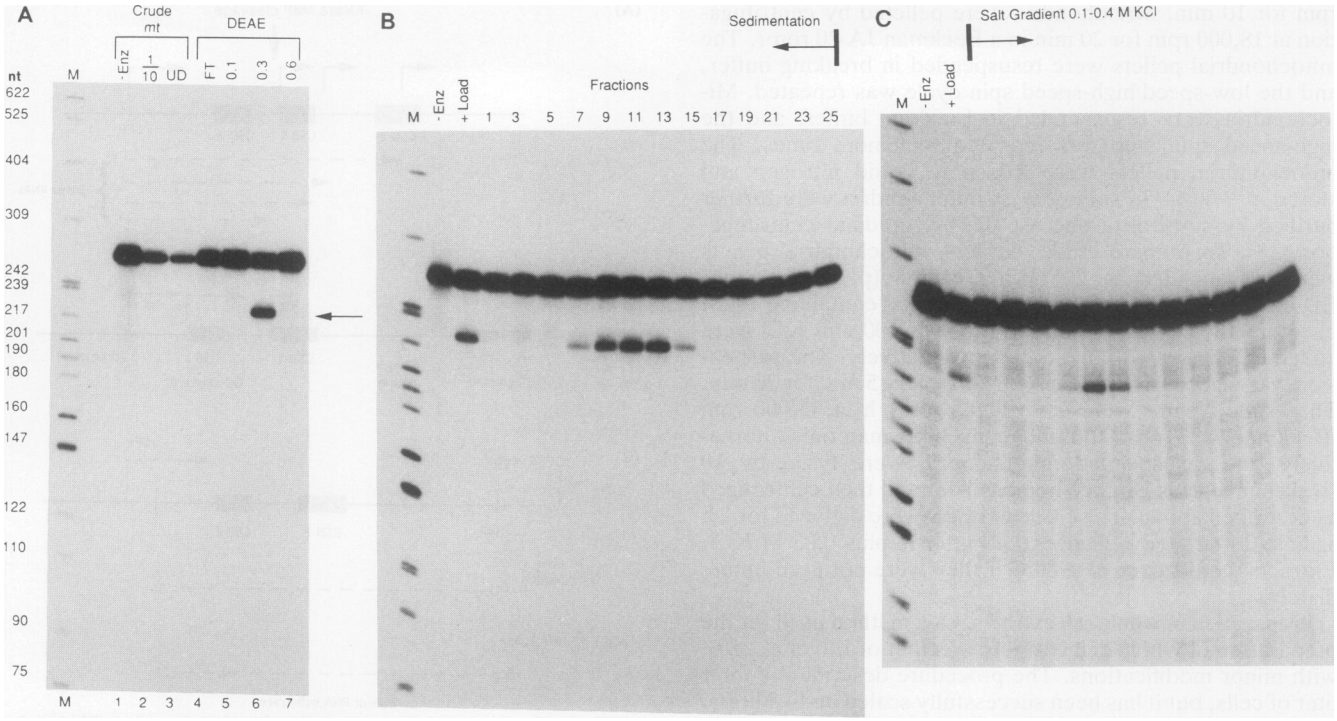

FIG. 2. Detection and partial purification of yeast RNase activity. (A) Detection of yeast cleavage activity in crude mitochondrial lysates and after DEAE-Sephacel chromatography. A salt-detergent lysate of yeast mitochondria was prepared as described in Materials and Methods. In vitro processing reactions using the yeast *ori5* substrate (Fig. 1C) were assayed as described in Materials and Methods. Lane M contains *Hpa*II fragments of pBR322 as molecular weight size standards. Lanes: 1, minus-enzyme control (−Enz), no mitochondrial proteins added; 2, 1/10 dilution of a crude mitochondrial extract (1 μg of protein); 3, 1 μl of the undiluted (UD) crude extract (10 μg of protein); 4, DEAE-Sephacel flowthrough (FT) (4.1 μg of protein); 5, 0.1 M KCl wash (3.0 μg of protein); 6, 0.3 M KCl wash (1.1 μg of protein); 7, 0.6 M KCl wash (2.2 μg of protein). The arrow indicates the generation of the 225-nt processed RNA. (B) Purification of the yeast RNase by glycerol gradient sedimentation. After fractionation of yeast mitochondrial extracts on DEAE-Sephacel, active 0.3 M KCl fractions were pooled and dialyzed against 50 mM KCl. A portion was subjected to glycerol gradient centrifugation (10 to 30% glycerol). Every other fraction was tested for cleavage activity. Markers (lane M) were *Hpa*II-digested pBR322 DNA; lane −Enz is a minus-enzyme control; lane +Load is a positive enzyme control assay of starting material loaded onto the glycerol gradient. (C) Fractionation of yeast RNase on an FPLC Mono Q column. Pooled active fractions were loaded onto an FPLC Mono Q column preequilibrated with 0.05 M KCl, washed with 0.1 M KCl, and eluted with a linear gradient of 0.1 to 0.4 M KCl. Every other fraction was tested for cleavage activity; only the linear salt gradient portion of the column elution is shown. Lanes: M, *Hpa*II-digested pBR322 DNA; −Enz, minus-enzyme control; +Load, active starting material.

liters containing 0.4 to 1.0 mg of protein was loaded onto a 4.0-ml gradient and centrifuged for 5 to 6 h at 60,000 rpm in a Beckman SW60Ti rotor. Alternatively, 200 μl of extract containing 1.0 to 1.5 mg of protein was centrifuged for 24 h at 40,000 rpm in a Beckman SW41Ti rotor. Gradients were fractionated from the bottom by pumping, and every other fraction was tested for cleavage activity. Active fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0)–0.05 M KCl–0.5 mM EDTA–1 mM DTT–10% glycerol. For fast protein liquid chromatography (FPLC), active fractions were loaded onto a Mono Q column equilibrated in the buffer described above at 0.5 ml/min. The column was washed with 10 ml of starting buffer and then with 10 ml of starting buffer plus 0.1 M KCl. The column was eluted with a 10-ml linear gradient of 0.1 to 0.4 M KCl. Every other fraction was tested for cleavage activity, and active fractions were pooled. When necessary, protein fractions were frozen in liquid nitrogen and stored at −70°C. Protein concentrations were determined by the modified dye-binding method of Read and Northcote (25).

Treatment of yeast activity with nucleases and proteinase K. For micrococcal nuclease digestion, the yeast extract was preincubated at 37°C for either 0 or 20 min in a 10-μl volume containing either $CaCl_2$ (2 mM) or micrococcal nuclease (5 U) or both together. After incubation, 1 μl of 0.1 M EGTA (pH 8.0) was added to terminate the nuclease digestion, and the mixture was mixed and then allowed to sit for 3 min at room temperature. Then 15 μl of a mix containing 5 μl of 5× reaction buffer (0.1 M Tris-HCl [pH 8.0], 50 mM $MgCl_2$, 5 mM DTT, 0.25 M KCl), 10 U of RNAguard, and 4,000 cpm of labeled substrate RNA was added. The reaction mixture was incubated for 30 min more at 37°C to assay for remaining cleavage activity, the reaction was terminated, and products were analyzed as described above. For DNase I treatment, yeast extracts were preincubated in 10 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and various amounts of DNase I. After 30 min at 37°C, 15 μl of a mix containing 5 μl of 5× reaction buffer (see above), 10 U of RNAguard, and 4,000 cpm of labeled RNA substrate was added, and the mixture was incubated for an additional 30 min at 37°C prior to analysis of cleavage products. For proteinase K treatment, yeast extracts were preincubated in a 10-μl volume containing 50 mM Tris-HCl (pH 8.0) and various amounts of proteinase K. After 20 min at 30°C, 15 μl of reaction buffer (see above) was added, reaction mixtures were incubated for

30 min at 37°C, and the products were analyzed as described above.

Mapping of in vitro cleavage site. The enzymatic sequence ladders used for mapping the in vitro cleavage site were generated by incubating 20,000 cpm of the p64/HS40 *ori*5/4.4 145-nucleotide (nt) substrate RNA and 5 μg of carrier tRNA in 5 μl of reaction buffer containing 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 7 M urea, and 0.025% each xylene cyanole and bromphenol blue for 15 min at 50°C with the following enzymes: 1 U (Pharmacia) or 0.01 or 0.005 U (Bethesda Research Laboratories) of RNase T_1, 1 U of RNase U2, and 1 U of RNase PhyM. The reaction was terminated by placing tubes in an ice bath, followed by immediate loading onto a 6% acrylamide–7 M urea gel. Partial alkaline hydrolysis ladders were generated by heating 40,000 cpm of substrate RNA in 20 μl of 50 mM $NaHCO_3$ (pH 9.1)–1 mM EDTA. After incubation at 90°C for 10 min, the sample was lyophilized and resuspended in 5 μl of the reaction buffer described above.

RESULTS

Identification and partial purification of a yeast endoribonuclease from mitochondrial and whole cell extracts. Similar organizational features of the mammalian mtDNA leading-strand replication origin and the putative yeast wild-type mtDNA origin are aligned in Fig. 1. Mouse or human RNase MRP cleaves mitochondrial transcripts immediately adjacent to CSB II (4, 28) (Fig. 1A). To determine whether yeast cells contained an RNase activity that could perform a specific endonucleolytic cleavage at the GC cluster C/CSB II region (Fig. 1B), a 3′-end-labeled yeast mtRNA substrate spanning the *ori*5 sequence was prepared as shown in Fig. 1C. When a crude mitochondrial lysate was incubated with the 3′-end-labeled substrate, a processed RNA species of ~225 nt was observed (Fig. 2A). This corresponds to cleavage immediately 5′ to the GC cluster C/CSB II element. However, the reaction was inefficient and the substrate was subject to degradation or loss, probably as a result of the presence of other nonspecific nucleases or RNA-binding proteins. To purify this activity, different fractionation procedures were performed. The mitochondrial extract was initially fractionated on DEAE-Sephacel; the cleavage activity bound to this column at 0.1 M KCl and was eluted at 0.3 M KCl (Fig. 2A). This chromatography procedure increased the efficiency of the cleavage reaction, and the substrate remained more intact. The active fractions were pooled and further purified over a 10 to 30% glycerol gradient (Fig. 2B), a second DEAE column, and an FPLC Mono Q column (Fig. 2C). We estimated that this sequence of steps results in at least a 100-fold purification of activity. During the course of these experiments (as was previously observed with mouse and human RNase MRP activities [2, 17, 28]), it became apparent that there was a substantial amount of activity located outside the mitochondria. We therefore began using yeast whole cell extracts as the convenient source of this endonuclease. Experiments demonstrated that the enzyme prepared from whole cell extracts exhibited the same behavior over DEAE-Sephacel, glycerol gradients, and FPLC Mono Q columns as did enzyme prepared from mitochondrial extracts (data not shown).

Requirements for enzymatic cleavage activity. Initially, the yeast processing reactions were carried out by using the optimal conditions previously determined for mouse RNase MRP (4). We subsequently assayed yeast cleavage activity under a variety of conditions to determine optimal ionic strength, monovalent and divalent cation requirements, pH and temperature optima, and the effect of tRNA and spermidine on the cleavage reaction (data not shown). The yeast activity does not require monovalent ions but is stimulated approximately twofold at 25 to 50 mM KCl. Higher salt concentrations (100 mM KCl) were extremely inhibitory. In contrast, the activity shows an absolute requirement for divalent ions. The enzyme is active in $MgCl_2$, with an optimum concentration of 5 to 10 mM. Other divalent cations tested ($MnCl_2$, $CaCl_2$, and $ZnCl_2$) could not substitute for $MgCl_2$. The enzyme is active over a broad pH range (6.5 to 8.5), with maximal activity occurring between pH 7.5 and 8.0. The enzyme is slightly active at 25°C and is maximally active at approximately 37°C. Although still moderately active at 42°C, raising the temperature to 50°C or above results in almost complete loss of activity. *E. coli* tRNA added to the reaction at 2 μg/ml almost completely inhibits the reaction; this inhibitory effect is purification stage dependent. *E. coli* tRNA at this concentration has no effect on crude extracts or fractions purified through one step. Extracts must be purified through two or three steps to observe tRNA inhibition. Spermidine had a slight stimula-

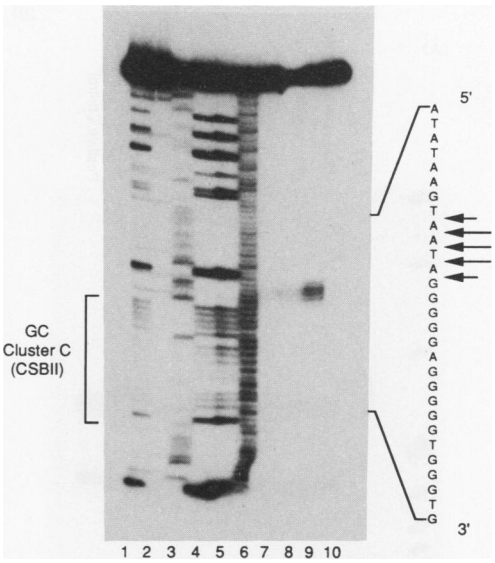

FIG. 3. Precise mapping of the in vitro cleavage site. The products of in vitro processing reactions were sized against enzymatic sequence ladders of a 145-nt pCp-labeled precursor RNA substrate to determine the nucleotide positions of cleavage by yeast enzyme isolated from whole cell and mitochondrial extracts. The RNases used to generate the sequence ladders were as follows: lanes 1, 4, and 5, RNase T_1 (cleaves after G); lane 2, RNase U2 (cleaves after A); lane 3, RNase PhyM (cleaves after A and U); lane 6, partial alkaline hydrolysis ladder; lane 7, in vitro processing reaction carried out with yeast enzyme isolated from whole cell extracts (0.2 μg of protein) after DEAE-Sephacel and glycerol gradient centrifugation; lane 8, a different whole cell enzyme preparation (0.22 μg of protein) after DEAE-Sephacel and two glycerol gradients; lane 9, yeast enzyme isolated from mitochondrial extracts (1.2 μg of protein) and purified through two glycerol gradients; lane 10, minus-enzyme control. Cleavage products are seen in modest abundance in this exposure in order to enable visualization of individual species. The bracketed area on the left indicates the position of the GC cluster C/CSB II region of the *ori*5 substrate. The sequence of the substrate surrounding the cleavage points (arrowheads) is shown. The length of the arrow represents the relative abundance of cleavage at that bond.

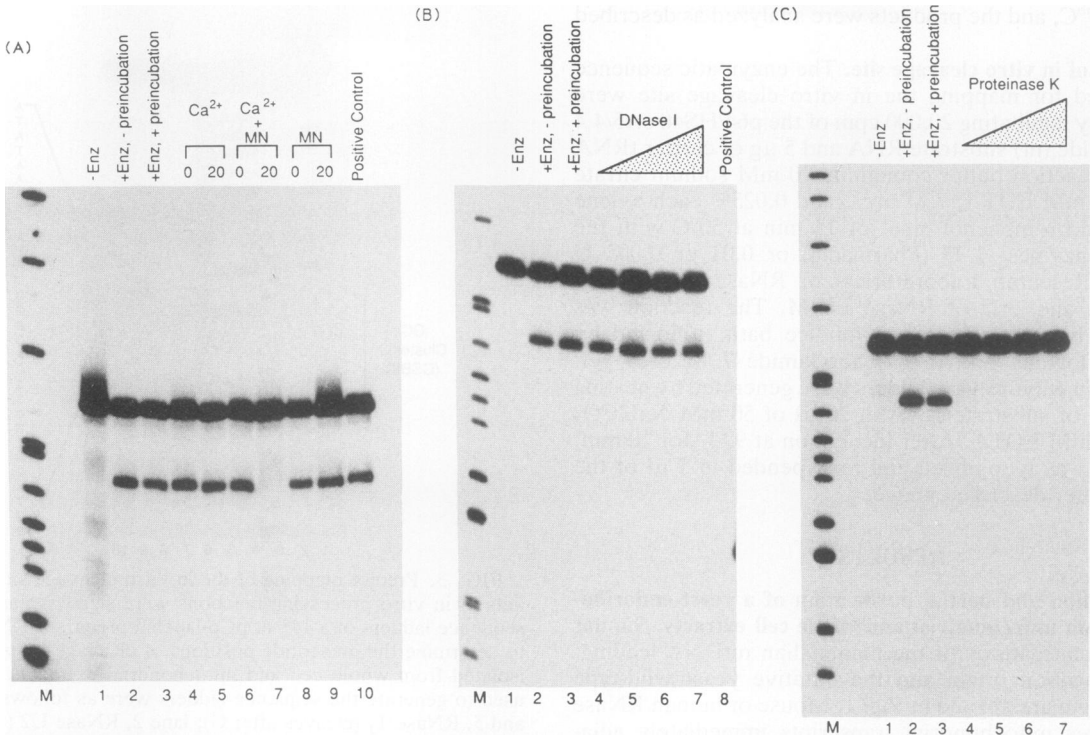

FIG. 4. Treatment of yeast enzyme with various nucleases. (A) Inactivation of yeast enzyme with micrococcal nuclease. Yeast enzyme was incubated with $CaCl_2$ alone (lanes 4 and 5), with $CaCl_2$ and micrococcal nuclease (MN) (lanes 6, 7), or with micrococcal nuclease alone (lanes 8 and 9) for either 0 or 20 min, as indicated. Nuclease digestion was terminated by the addition of EGTA, and the cleavage activity remaining was assayed. Control lanes show that a 20-min preincubation alone (lane 3) had no significant effect on cleavage activity (compared with no preincubation [lane 2]). Lane 10 (positive control) shows that after prior treatment with micrococcal nuclease and $CaCl_2$ and termination of digestion by addition of EGTA, the cleavage activity can be regained by adding back a fresh aliquot of enzyme. Lane M contains *Hpa*II-digested pBR322 DNA. Lane 1 is a minus-enzyme control (−Enz). (B) Insensitivity of yeast enzyme to DNase I treatment. Yeast enzyme extract was preincubated for 30 min in buffer containing 10 mM $MgCl_2$ and various amount of DNase I and then tested for substrate cleavage activity. Lane 1 is a minus-enzyme control (−Enz). Additional control lanes show that a 30-min preincubation alone, in the absence of DNase I (lane 3), had no significant effect on cleavage activity compared with extract with no preincubation (lane 2). Lane 8 is a positive control demonstrating that the DNase I used in this study was active; it contains ^{32}P-labeled *Hpa*II-digested pBR322 markers that were incubated together with 2 μg of cold plasmid DNA and 20 μg of *E. coli* tRNA in the presence of 1 U of DNase I. In lanes 4 to 7, yeast enzyme was preincubated in the presence of increasing amounts of DNase I (0.01 U [lane 4], 0.1 U [lane 5], 1 U [lane 6]; 10 U [lane 7]) and then assayed for cleavage activity. Lane M, *Hpa*II-digested pBR322 DNA. (C) Inactivation of yeast enzyme by proteinase K treatment. Yeast enzyme was assayed for cleavage activity with or without prior incubation with proteinase K. Lane 1 is a minus-enzyme control (−Enz). Control lanes show that a 20-min preincubation alone, in the absence of proteinase K, had no significant effect on cleavage activity (lane 3) compared with extract with no preincubation (lane 2). In lanes 4 to 7, yeast enzyme was preincubated in the presence of increasing amounts of proteinase K (0.1 mg [lane 4], 0.5 mg [lane 5], 2 mg [lane 6], 4 mg [lane 7]) and then assayed for cleavage activity. Lane M contains *Hpa*II-digested pBR322 DNA.

tory effect at lower concentrations (0.5 to 1.0 mM) but inhibited the reaction at higher concentrations. All of these results are very similar to those obtained for mouse RNase MRP (4).

Mapping of the in vitro cleavage site. The size of the 3′-labeled reaction product was ~225 nt, indicating that cleavage of the *ori*5 substrate occurred at the 5′ end of the GC cluster C/CSB II region. The 5′-end-labeled substrate is also cleaved endonucleolytically (data not shown). To determine the in vitro cleavage site more precisely, the products of a processing reaction were sized against the enzymatic sequence ladders of the identical precursor substrate. The results shown in Fig. 3 demonstrate that cleavage occurs at five adjacent nucleotides, 5′-TAATA-3′ (three major and two very minor sites), located just upstream of the GC cluster C/CSB II region of the yeast *ori*5 sequence. This multinucleotide staggering of cleavage sites for the yeast enzyme is similar to that observed for mouse and human RNase MRPs (2, 4). The cleavage pattern obtained is the same whether the enzyme was purified from whole cell extracts or isolated mitochondria.

Yeast RNase activity requires both an RNA and a protein component. Both mouse and human RNase MRPs are ribonucleoprotein particles requiring an RNA and protein species for enzymatic cleavage activity (5, 28). To investigate whether the yeast enzyme required a nucleic acid component, the activity was assayed after digestion with micrococcal nuclease, a nuclease that requires Ca^{2+} as a cofactor and degrades both RNA and DNA. As shown in Fig. 4A, preincubation of the yeast enzyme for 20 min in the presence of Ca^{2+} alone or micrococcal nuclease alone had no significant effect on cleavage activity (lanes 5 and 9). However, preincubation in the presence of both micrococcal nuclease and Ca^{2+} resulted in complete inactivation of the cleavage activity (lane 7). Lane 10 shows that the activity can be regained by adding back a fresh aliquot of enzyme extract,

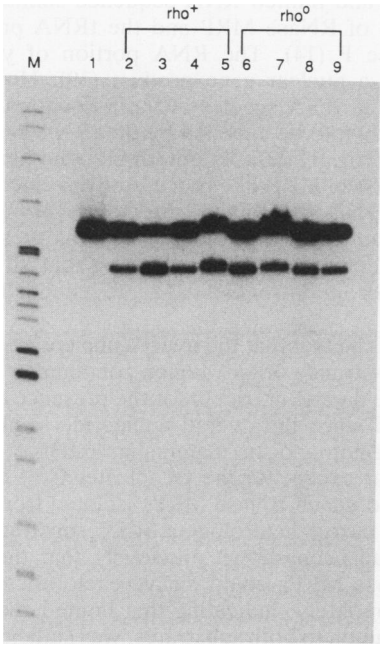

FIG. 5. Presence of yeast site-specific RNase in both [*rho*$^+$] and [*rho*^{0}] strains. Whole cell extracts were prepared from two yeast strains containing mtDNA ([*rho*$^+$]) and two strains lacking mtDNA ([*rho*^{0}]) and tested for their ability to cleave the *ori*5 substrate. Lanes: M, *Hpa*II-digested pBR322 DNA; 1, minus-enzyme control; 2 and 3, 1 and 5 μl of extract from strain MH41-7B; 4 and 5, 1 and 5 μl of extract from strain S288C; 6 and 7, 1 and 5 μl of extract from the strain containing a disrupted mtRNA polymerase gene (15); 8 and 9, 1 and 5 μl of extract from [*rho*^{0}] tester strain D243-4A 18.

indicating that the observed inactivation is not due to the presence of an inhibitor generated during micrococcal nuclease digestion. These results indicate that a nucleic acid species is required for activity. Figure 4B shows that prior treatment of the enzyme fraction with DNase I does not eliminate activity, consistent with the required component being an RNA rather than a DNA species. Figure 4C shows that prior treatment of the enzyme with proteinase K results in complete inactivation. These results are consistent with the yeast enzyme being a ribonucleoprotein particle that requires both RNA and protein components for cleavage activity.

All components necessary for cleavage activity in vitro are nuclear encoded. To determine whether any essential parts of this ribonucleoprotein enzyme were encoded by the mitochondrial genome, whole cell extracts were prepared from two yeast strains lacking mtDNA ([*rho*^{0}]) and tested for their ability to cleave the yeast *ori*5 substrate. Figure 5 shows that enzyme extracts prepared from wild-type ([*rho*$^+$]) strains, as well as both [*rho*^{0}] strains, can efficiently cleave the *ori*5 substrate. These results demonstrate that all components of the yeast enzyme necessary for in vitro cleavage are nuclear gene products.

In vitro cleavage assays using heterologous substrates. The overall nucleotide sequences of the leading-strand origin region of mouse and human mtDNAs are quite different except for the three short evolutionarily conserved sequence blocks termed CSBs I, II, and III. Human mtRNA substrate can be appropriately cleaved by mouse RNase MRP, and human RNase MRP cleaves the mouse mtRNA substrate at the same position as that of the homologous mouse enzyme; this finding indicated that limited sequence information was sufficient for proper recognition of the cleavage site (2, 4, 27). Site-directed mutational analysis of the mouse substrate demonstrated that CSBs II and III are required for both accurate and efficient cleavage (2).

Since the overall nucleotide sequence of the yeast *ori*5 region and the mammalian mtDNA origin region are also very different except for the presence of the GC cluster C/CSB II element (Fig. 6A) and a potential CSB I cognate, it was of interest to determine whether the yeast RNase activity could cleave the mammalian mtRNA substrate. Figure 6B demonstrates that the yeast enzyme cleaves human substrate (lanes 2, 4, and 5) in the same manner as does human RNase MRP (lane 3). Both RNases generate a 190-nt product resulting from cleavage immediately 5′ to the CSB II element. The yeast enzyme also cleaves a standard mouse substrate at the CSB II element (data not shown). Several point mutations in the CSB II region that affect the ability of mouse RNase MRP to cleave such mutated mouse substrates were tested (2); in these cases, cleavage by the yeast RNase was also affected (data not shown). It has been previously shown that the yeast *ori*5 region is a suitable RNA substrate for cleavage near the CSB II-like element by either human or mouse RNase MRP (27). Together, these results suggest that the CSB II-like element is playing a role in cleavage site selection for the yeast RNase as well.

DISCUSSION

A subset of yeast mitochondrial promoters is located immediately adjacent to putative origins of replication; as such, they have been implicated in priming of DNA replication. A common feature of these replication origins is the presence of a short guanosine-rich region (GC cluster C/CSB II-like element) in the priming strand downstream of the transcription start site (1, 10, 13, 26). This situation is similar to that for the vertebrate mtDNA leading-strand origin and suggests possible functional relationships between the yeast *ori*/*rep* and vertebrate origin systems. Since mammalian cells contained an RNA processing activity that could cleave RNA sequence at CSB II, we investigated whether yeast cells contained any RNase with similar specificity.

This report describes the identification and partial characterization of a yeast RNase that cleaves the yeast *ori*5 mtRNA sequence in a site-specific manner immediately adjacent to the vertebrate CSB II sequence homolog, GC cluster C. This activity can be isolated from mitochondrial fractions as well as whole cell extracts and exhibits similar purification behavior in each case and in comparison with mammalian RNase MRP. The position of the in vitro RNA cleavage site was determined, demonstrating that the yeast enzyme cleaves the yeast *ori*5 sequence predominantly at three adjacent nucleotides located immediately upstream of the GC cluster C/CSB II element (Fig. 6A). These nucleotides are exactly at the mapped position of linkage between RNA and DNA in nucleic acid isolated from mitochondria of a hypersuppressive yeast strain containing a mtDNA *ori*5 sequence (1). The multinucleotide staggering of cleavage points for the yeast enzyme is similar in breadth and position to that observed for mammalian RNase MRPs. Both mouse and human RNase MRPs are ribonucleoprotein particles; this study shows that the yeast RNase is likely a similar ribonucleoprotein that requires both RNA and protein components for cleavage activity.

In the case of mammalian cells, there is an antigenic

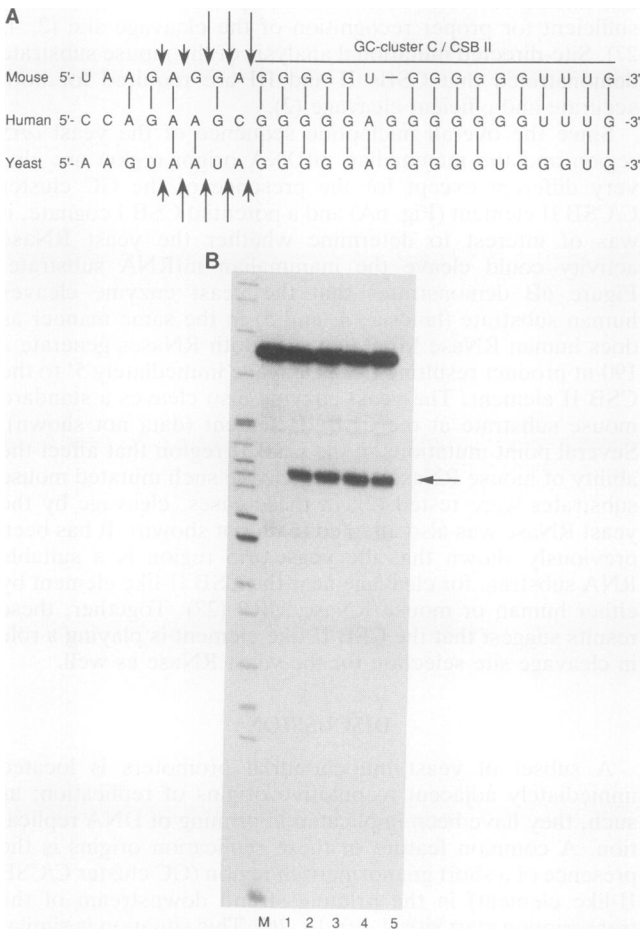

FIG. 6. In vitro cleavage assay using a heterologous substrate. (A) Comparison of GC cluster C/CSB II sequences and cleavage sites in mouse, human, and yeast cells. Sequence alignment of the CSB II element in the mouse and human mtDNA leading-strand origin region (4) and the GC cluster C/CSB II element of the yeast mtDNA *ori*5 region (1) is shown. The horizontal line above the sequences denotes these elements. Nucleotide identities are indicated by vertical lines. The dash indicates the absence of a nucleotide at that position. Cleavage sites are indicated by the vertical arrows. The three longer arrows pointing downward denote the major points of cleavage by human or mouse RNase MRP on the human or mouse RNA sequence shown. The three longer arrows pointing upward denote the major points of cleavage by the yeast RNase on the yeast RNA sequence shown (Fig. 3). The yeast site of cleavage is a reported site of linkage of RNA to DNA in mitochondrial nucleic acid (1). The shorter arrows denote minor cleavage points. (B) In vitro cleavage assay using a human mtRNA substrate. The substrate was a 320-nt pCp-labeled human mtRNA sequence generated from plasmid pK408 (see Materials and Methods). Lanes: M, *Hpa*II-digested pBR322 DNA; 1, minus-enzyme control; 2, yeast enzyme from whole cell extracts (0.1 μg of protein) partially purified over a DEAE-Sephacel column and a 10 to 30% glycerol gradient; 3, human RNase MRP from extracts (0.5 μg of protein) partially purified over a 10 to 30% glycerol gradient; 4, yeast enzyme from a different whole cell extract (0.2 μg of protein) partially purified over a DEAE-Sephacel column, a glycerol gradient, and a second DEAE-Sephacel column; 5, yeast enzyme from a mitochondrial extract (1.0 μg of protein) partially purified over two 10 to 30% glycerol gradients. The arrow indicates the position of the 190-nt radioactive RNA product generated in these reactions.

relationship and limited RNA sequence similarity between components of RNase MRP and the tRNA processing enzyme RNase P (14). The RNA portion of yeast nuclear RNase P is a nuclear gene product (19). However, in *S. cerevisiae*, the RNA species of the mitochondrial RNase P has been shown to be encoded by the tRNA synthesis locus of mtDNA (16, 21, 22). To determine whether any part of this yeast RNase MRP-like processing enzyme was encoded by yeast mtDNA, extracts prepared from [*rho*^{0}] strains were tested. It was found that the activity was present in [*rho*^{0}] yeast cells lacking mtDNA, indicating that all components necessary for in vitro cleavage are encoded by nuclear genes.

In view of the fact that the overall nucleotide sequences of the leading-strand origin region of human and mouse mtDNAs are divergent except for the presence of CSBs I, II, and III, and since the overall nucleotide sequences of the yeast *ori*5 region and the mammalian mtDNA origin region are dissimilar except for the GC cluster C/CSB II element, the degree to which RNase MRPs isolated from these three species can cleave heterologous RNA substrates is of relevance. It had been shown previously that the mouse and human RNase MRPs could cleave each other's RNA substrate appropriately, indicating that limited sequence information, common to both substrates, was sufficient for proper recognition of the cleavage site (2, 4, 27). Prior experiments have also shown that both mouse and human enzymes are capable of processing yeast *ori*5 RNA substrate with low efficiency (27); reduced cleavage is most likely due to the absence of a CSB III efficiency element in the yeast substrate (2). We extended this study to the yeast enzyme, which was capable of cleaving mammalian RNA substrates containing the leading-strand origin region of mtDNA. The yeast RNase cleaves human substrate in the same manner as does human RNase MRP.

The physical characteristics and reaction parameters of this yeast site-specific endoribonuclease argue strongly for its identity as yeast RNase MRP. A definitive assignment should come from an eventual identification and characterization of the RNA component of this yeast ribonucleoprotein. This would also provide a powerful system in which to investigate the role of RNase MRP in the nucleus, its mechanism of intracellular transport, and any exact requirements for RNase MRP in yeast mitochondria.

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REFERENCES

1. **Baldacci, G., B. Chérif-Zahar, and G. Bernardi.** 1984. The initiation of DNA replication in the mitochondrial genome of yeast. EMBO J. **3**:2115–2120.
2. **Bennett, J. L., and D. A. Clayton.** 1990. Efficient site-specific cleavage by RNase MRP requires interaction with two evolutionarily conserved mitochondrial RNA sequences. Mol. Cell. Biol. **10**:2191–2201.
3. **Chang, D. D., and D. A. Clayton.** 1985. Priming of human mitochondrial DNA replication occurs at the light-strand promoter. Proc. Natl. Acad. Sci. USA **82**:351–355.
4. **Chang, D. D., and D. A. Clayton.** 1987. A novel endoribonuclease cleaves at a priming site of mouse mitochondrial DNA

replication. EMBO J. **6**:409–417.
5. **Chang, D. D., and D. A. Clayton.** 1987. A mammalian mitochondrial RNA processing activity contains nucleus-encoded RNA. Science **235**:1178–1184.
6. **Chang, D. D., and D. A. Clayton.** 1989. Mouse RNAse MRP RNA is encoded by a nuclear gene and contains a decamer sequence complementary to a conserved region of mitochondrial RNA substrate. Cell **56**:131–139.
7. **Chang, D. D., W. W. Hauswirth, and D. A. Clayton.** 1985. Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA. EMBO J. **4**:1559–1567.
8. **Christianson, T.** 1983. Ph.D. thesis. University of Chicago, Chicago, Ill.
9. **Clayton, D. A.** 1982. Replication of animal mitochondrial DNA. Cell **28**:693–705.
10. **de Zamaroczy, M., G. Faugeron-Fonty, G. Baldacci, R. Goursot, and G. Bernardi.** 1984. The *ori* sequences of a wild-type yeast strain: number, location, orientation and structure. Gene **32**: 439–457.
11. **England, T. E., and O. C. Uhlenbeck.** 1978. 3-terminal labelling of RNA with T4 RNA ligase. Nature (London) **275**:560–561.
12. **Fangman, W. L., and B. Dujon.** 1984. Yeast mitochondrial genomes consisting of only A · T base pairs replicate and exhibit suppressiveness. Proc. Natl. Acad. Sci. USA **81**:7156–7160.
13. **Faugeron-Fonty, G., C. Le Van Kim, M. de Zamaroczy, R. Goursot, and G. Bernardi.** 1984. A comparative study of the *ori* sequences from the mitochondrial genomes of twenty wild-type yeast strains. Gene **32**:459–473.
14. **Gold, H. A., J. N. Topper, D. A. Clayton, and J. Craft.** 1989. The RNA processing enzyme RNase MRP is identical to the Th RNP and related to RNase P. Science **245**:1377–1380.
15. **Greenleaf, A. L., J. L. Kelly, and I. R. Lehman.** 1986. Yeast *RPO41* gene product is required for transcription and maintenance of the mitochondrial genome. Proc. Natl. Acad. Sci. USA **83**:3391–3394.
16. **Hollingsworth, M. J., and N. C. Martin.** 1986. RNase P activity in the mitochondria of *Saccharomyces cerevisiae* depends on both mitochondrion and nucleus-encoded components. Mol. Cell. Biol. **6**:1058–1064.
17. **Karwan, R., J. L. Bennett, and D. A. Clayton.** 1991. Nuclear RNase MRP processes RNA at multiple discrete sites: interaction with an upstream G box is required for subsequent downstream cleavages. Genes Dev. **5**:1264–1276.
18. **Lambowitz, A. M.** 1979. Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. **59**:421–433.
19. **Lee, J.-Y., and D. R. Engelke.** 1989. Partial characterization of an RNA component that copurifies with *Saccharomyces cerevisiae* RNase P. Mol. Cell. Biol. **9**:2536–2543.
20. **Lin, R.-J., A. J. Newman, S.-C. Cheng, and J. Abelson.** 1985. Yeast mRNA splicing *in vitro*. J. Biol. Chem. **260**:14780–14792.
21. **Miller, D. L., and N. C. Martin.** 1983. Characterization of the yeast mitochondrial locus necessary for tRNA biosynthesis: DNA sequence analysis and identification of a new transcript. Cell **34**:911–917.
22. **Morales, M. J., C. A. Wise, M. J. Hollingsworth, and N. C. Martin.** 1989. Characterization of yeast mitochondrial RNAse P: an intact RNA subunit is not essential for activity *in vitro*. Nucleic Acids Res. **17**:6865–6881.
23. **Parikh, V. S., M. M. Morgan, R. Scott, L. S. Clements, and R. A. Butow.** 1987. The mitochondrial genotype can influence nuclear gene expression in yeast. Science **235**:576–580.
24. **Promega.** 1989–1990. Protocols for *in vitro* transcription, p. 42–44. *In* Protocols and applications guide. Promega Corp., Madison, Wis.
25. **Read, S. M., and D. H. Northcote.** 1981. Minimization of variation in the response to different proteins of the blue G dye-binding assay for protein. Anal. Biochem. **116**:53–64.
26. **Schinkel, A. H., and H. F. Tabak.** 1989. Mitochondrial RNA polymerase: dual role in transcription and replication. Trends Genet. **5**:149–154.
27. **Topper, J. N., D. D. Chang, R. P. Fisher, and D. A. Clayton.** 1988. Initiation and processing of mitochondrial displacement-loop transcripts, p. 207–212. *In* T. Kelly and B. Stillman (ed.), Cancer cells 6. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Topper, J. N., and D. A. Clayton.** 1990. Characterization of human MRP/Th RNA and its nuclear gene: full length MRP/Th RNA is an active endoribonuclease when assembled as an RNP. Nucleic Acids Res. **18**:793–799.
29. **Wong, T. W., and D. A. Clayton.** 1985. In vitro replication of human mitochondrial DNA: accurate initiation at the origin of light-strand synthesis. Cell **42**:951–958.
30. **Wong, T. W., and D. A. Clayton.** 1986. DNA primase of human mitochondria is associated with structural RNA that is essential for enzymatic activity. Cell **45**:817–825.