Yeast site-specific ribonucleoprotein endoribonuclease MRP contains an RNA component homologous to mammalian RNase MRP RNA and essential for cell viability

Mark E. Schmitt and David A. Clayton

Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305-5427 USA

RNase MRP is a site-specific ribonucleoprotein endoribonuclease that cleaves RNA sequence complementary to mammalian mitochondrial origins of replication in a manner consistent with a role in primer RNA metabolism. The same activity in the yeast *Saccharomyces cerevisiae* has recently been identified; it cleaves an RNA substrate complementary to a yeast mitochondrial origin of replication at an exact site of linkage of RNA to DNA. We have purified this yeast enzyme further and detect a single, novel RNA of 340 nucleotides associated with the enzymatic activity. The single-copy nuclear gene for this RNA was sequenced and mapped to the right arm of chromosome XIV. The identity of the clone, as encoding the RNA copurifying with enzymatic activity, was confirmed by a match to the directly determined sequence of the RNA. The gene sequence also identified a 340-nucleotide RNA in total yeast RNA and in purified RNase MRP enzyme preparations. Inspection of the sequence of the yeast RNA revealed homologies to the RNA component of mouse RNase MRP, 49% overall with specific regions of much greater similarity. The flanking regions of the gene showed characteristics of an RNA polymerase II transcription unit, including a TATAAA box and a 7/8 match to the yeast cell cycle box UAS. The RNase MRP RNA gene was deleted by insertional replacement and found to be essential for cellular viability, indicating a critical nuclear role for RNase MRP.

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Previous studies utilizing mammalian cells have established the existence of a novel RNA processing activity that cleaves RNA at specific sites (Chang and Clayton 1987a,b; Topper and Clayton 1990a; Karwan et al. 1991). The enzyme is a ribonucleoprotein (RNP) endoribonuclease whose RNA component and the nuclear gene that encodes it have been characterized from mouse (Chang and Clayton 1987b, 1989) and human (Topper and Clayton 1990a; Yuan and Reddy 1991) cells. The enzyme has been termed RNase MRP (mitochondrial RNA processing) because it processes mitochondrial RNA (mtRNA) complementary to the leading-strand origin of replication of mouse and human mitochondrial DNA (mtDNA). The exact positions of cleavage are at or within several nucleotides of known sites of mtRNA and mtDNA nascent strand termini in the priming region for mtDNA replication. Both RNA and protein components of RNase MRP are required for enzymatic activity.

However, the majority of cellular RNase MRP activity can be isolated from nuclear fractions (Topper and Clayton 1990a,b; Karwan et al. 1991), and autoantibodies to RNase MRP have been used to establish its antigenic

relationship with RNase P and identity with the Th ribonucleoprotein (Gold et al. 1989). In turn, an immunocytochemistry approach to detecting the intracellular location of the Th antigen has revealed the nucleolus as the site of most abundant presence of antigen (Reimer et al. 1988). These data suggest that RNase MRP is playing a role in nuclear RNA metabolism in addition to its proposed function in mitochondrial primer RNA formation.

Efforts to provide definitive direct evidence for the required action of RNase MRP in priming mammalian mtDNA replication have been severely hampered by the very low abundance of mitochondrial enzymes in mammalian cells, the lack of an in vitro replication assay for leading-strand mtDNA replication, the absence of a transformation system for mammalian mitochondria, and the inability to manipulate and disrupt easily any nuclear gene for a prospective mammalian mitochondrial enzyme. Furthermore, strategies to learn the nature of enzyme action in the mammalian nucleus are limited in their genetic approaches.

Most of the aforementioned problems would be abrogated by using the yeast *Saccharomyces cerevisiae*. The

basic experimental advantages afforded by this organism are well known (Botstein and Fink 1988). Of particular importance for studies of mitochondrial biogenesis are the facts that the relative amount of mtDNA in the cell is at least an order of magnitude greater than for typical mammalian cells and that yeast cells are viable and easily propagated in the total absence of mtDNA. This permits the study of nuclear mutations that affect the maintenance of mtDNA apart from loss of function of nuclear metabolic events.

The recent finding that *S. cerevisiae* contains an RNase MRP activity renders it the system of choice for studying the intracellular roles of this enzyme (Stohl and Clayton 1992). Yeast RNase MRP was shown to be a RNP endoribonuclease with properties similar to mammalian RNase MRPs. These include the ability to cleave both mammalian and yeast mtRNA sequences at the same primary locations (Stohl and Clayton 1992) and, in the case of yeast mtRNA, exactly at a singular mapped site of RNA to DNA synthesis (Baldacci et al. 1984).

Here, we report the identification of the yeast RNase MRP RNA component and the sequence, characterization, and chromosomal location of its nuclear gene. Deletion of the nuclear gene for yeast RNase MRP RNA results in loss of cell viability, indicating an important nuclear role for the enzyme.

Results

Purification of the yeast RNase MRP activity

Our previous numerous attempts at purifying yeast RNase MRP resulted in enzyme preparations with high specific activity but containing only small degraded RNAs. However, these preparations were inactivated by micrococcal nuclease, indicative of the presence of an essential RNA component (Stohl and Clayton 1992). This is similar to the situations seen for both the yeast nuclear and mitochondrial RNase P enzymes (Lee and Engelke 1989; Morales et al. 1989). Because of the high abundance of this enzyme in the yeast nucleus (Stohl and Clayton 1992), a whole-cell extract was chosen as the starting material for further purification. Several modifications of previous extraction procedures were used: The preparation of nuclei was avoided owing to the inefficiency of their isolation from yeast; glycerol and EDTA were included in the buffers to stabilize the enzymatic activity and to inhibit nuclease digestion of the enzymatic RNA component by endogenous nucleases, respectively; most important, a low salt extraction, 150 mM KCl, was used to isolate selectively RNase MRP and to avoid copurification of the majority of small nucleolar RNPs (snoRNPs) (Schimmang et al. 1989).

This whole-cell extract was then subjected to batch DEAE-Sephacel treatment under conditions where most of the protein is eluted in the flowthrough fraction and most of the splicing small nuclear RNPs (snRNPs) remain bound to the resin (Riedel et al. 1986). This initial step also removes the majority of exonucleases that are prevalent in yeast. The step elution from the Bio-Rex 70

column provides a further enrichment, as most proteins fail to bind to both cation and anion exchange resins. A final glycerol gradient centrifugation does not provide a significant increase in specific activity but does remove most of the tRNAs that otherwise present a prominent background in subsequent [³²P]pCp labeling. This separation is effective because of the large size of the RNase MRP complex, which is estimated to be at least 600,000 daltons in mammalian cells (J.L. Bennett and D.A. Clayton, unpubl.).

An activity profile through the purification scheme is shown in Figure 1A. A transcript complementary to the yeast mtDNA replication origin *ori5* was used as the RNA substrate (de Zamaroczy et al. 1984). The amounts of activity seen in the Bio-Rex 70 elution and the final 15–30% gradient peak are underestimated; this is largely the result of saturation of the assay. A 10-fold dilution of these fractions gives a comparable amount of enzymatic cleavage (data not shown).

Identification of copurifying RNAs

RNAs from the peak fractions of the glycerol gradient were 3'-end labeled with [³²P]pCp and RNA ligase (Fig. 1B). Two RNAs, of 340 and 125 nucleotides, were prominent. A similar pattern was also seen when the yeast cells were labeled with [³²P]orthophosphate before breakage of the cells; however, ~80% of the isotope was then incorporated into the 340-nucleotide RNA. Neither of these two major RNA species was precipitated by trimethylguanosine antibodies (data not shown) (Riedel et al. 1986), indicating that these species were not capped at their 5' ends.

The two RNAs were excised from a gel and subjected to enzymatic sequencing from the 3' end (data not shown). The 125-nucleotide RNA was identical to the published sequence of 5S rRNA (Skryabin et al. 1984). The 340-nucleotide RNA proved to be heterogeneous at its 3' end by 1 nucleotide; these two species were equal in abundance in the enzyme preparations. Despite this situation, an interpretable sequence was determined that did not match any prior sequenced DNA elements in the yeast GenBank data base, including U3 (Hughes et al. 1987) and the nuclear RNase P (Lee and Engelke 1989) RNAs, which are of similar size.

Cloning of the gene for the yeast RNase MRP RNA

Radiolabeled cDNA was synthesized from the gel-purified 340-nucleotide RNA. This produced a high specific activity probe that was used to screen a randomly sheared yeast genomic library (see Materials and methods; Elledge et al. 1991). Two clones that hybridized with the probe were identified and found to contain overlapping yeast DNA inserts (Fig. 2A). A 1.1-kb *Eco*RI fragment of pMES125 was found to be inclusive of the hybridizing region. This fragment was subcloned into the vector pBluescript II KS+ for further analysis. This 1.1-kb fragment hybridized to a single band on a genomic

Figure 1. Purification and analysis of the yeast RNase MRP. **(A)** RNase MRP activity of different fractions through the purification steps. Fractions assayed include the clarified 150 mM KCl whole-cell extract, the flowthrough of DEAE-Sephacel, the 250 mM KCl elution from DEAE-Sephacel, the flowthrough of Bio-Rex 70, the 500 mM KCl elution from Bio-Rex 70, and the activity peak from a subsequent 15–30% glycerol gradient. The largest species in all lanes is the substrate: a 147-nucleotide RNA transcript from the yeast mitochondrial *ori5* region (de Zamaroczy et al. 1984) that has been 3'-end-labeled with [³²P]pCp. Cleavage products were separated on a 6% polyacrylamide 7 M urea gel (Chang and Clayton 1987a). The major cleavage product of 103 nucleotides corresponds to the site of cleavage reported by Stohl and Clayton (1992). Size standards are pBR322 DNA digested with *Hpa*II. The amount of protein added to each assay is indicated. **(B)** RNA detected in the purified fraction. The peak enzymatic fraction from the glycerol gradient was deproteinized, and RNAs were labeled with [³²P]pCp and RNA ligase, and fractionated by electrophoresis on a 6% denaturing polyacrylamide gel (Chang and Clayton 1987a). The identity of the 5S rRNA was confirmed by RNA sequencing (Skryabin et al. 1984; data not shown).

Southern, indicating that the DNA region encompassing this fragment was unique in the yeast genome. We have designated this gene *NME1* for nuclear mitochondrial processing endoribonuclease 1. Using a yeast ordered cosmid library, the *NME1* gene was placed on the right arm of chromosome XIV between *TOP2* and *SUF10*.

Sequence of the *NME1* gene

The complete sequence of the 1.1-kb *Eco*RI fragment was determined (see Materials and methods; Fig. 2B). Within the DNA sequence of this fragment, a 40-bp region that matched the 3' end of the determined RNA sequence was identified, confirming that this gene corresponds to that of the RNA isolated from RNase MRP enzyme preparations. A poly(T) tract was found at an expected distance from the RNA region sequenced. The 5' region of the *NME1* gene contains a presumptive TATAAA box at –96 nucleotides from the transcriptional start site (Parker et al. 1988). Also, the *HO* endo-

nuclease cell cycle box upstream activating sequence (UAS), with a 1-bp mismatch [7/8], is present (Nasmyth and Shore 1987). The flanking regions contain no putative reading frame of >36 amino acids. Also, no potential neighboring tRNA genes, δ elements, or Ty elements were found.

Analysis of the *NME1* RNA transcript

To confirm the expression of an RNA from this region of DNA, Northern analysis was performed. Both total RNA from cells grown on different carbon sources and purified 340-nucleotide RNA from an RNase MRP enzyme preparation were examined. A radioactive probe generated from the 1.1-kb fragment was hybridized with either the total RNA sample or the RNA from the enzyme preparation (Fig. 3A). A single full-length species of 340 nucleotides was detected in all lanes, reconfirming the isolation of the gene encoding the 340-nucleotide RNA. No potential precursor RNA was observed, as was also the case for yeast nuclear RNase P RNA (Lee and Engelke

Figure 2. Cloning and sequence determination of *NME1*, the gene encoding the RNA component of yeast RNase MRP. (A) Restriction map and sequencing strategy of overlapping genomic clones for *NME1*. Positive λ clones hybridizing to the probe were excised into plasmids using the *cre-lox* system (Elledge et al. 1991) and analyzed by restriction mapping. The region that hybridizes with the probe is enlarged. The arrow above the gene represents the direction of transcription. The arrows below the gene represent the sequencing strategy. Restriction sites are *EcoRI*, (E); *XhoI*, (X); *HindIII*, (H); *KpnI*, (K); *NcoI*, (N). Asterisks (*) denote the ends of the random genomic inserts where *EcoRI*-*XhoI* linkers have been ligated (Elledge et al. 1991). (B) Nucleotide sequence of the *NME1* gene. The sequence was determined using the Sequenase Kit (U.S. Biochemical). The start of transcription is numbered as +1. Relevant restriction sites are shown. The sequence of the RNA is shown in uppercase letters above the DNA sequence. The sequenced RNA region is underlined. The putative TATAAA box (Parker et al. 1988) and a 7/8 match to the cell cycle box UAS (Nasmyth and Shore 1987) are underlined. The EMBL accession number is Z14231.

1989). The RNA present in the enzyme preparation was full-length as well. No glucose repression of expression of this RNA was found when comparing RNA from cells grown on 10% dextrose to cells grown on 2% dextrose or on 4% ethanol and 3% glycerol (data not shown); RNA levels were comparable (Fig. 3A).

The 3' and 5' termini of the expressed RNase MRP RNA were mapped by RNA protection assays (Fig. 3B; Sambrook et al. 1989). A 569-nucleotide complementary RNA probe, generated from the region 3' of the *KpnI* site internal to the *NME1* gene, was hybridized with total cellular RNA and with purified 340-nucleotide RNA from an RNase MRP enzyme preparation prior to single-stranded nuclease digestion. A doublet at ~146 nucleotides was detected in all three experimental lanes and did not appear in either of the control lanes (probe alone and tRNA). This pair of RNA species is consistent with the RNA sequencing data that showed RNase MRP RNA to possess a 1 nucleotide heterogeneity at the 3' end. This places the 3' end of the RNA at the last two nucleotides before the poly(T) stretch. Such poly(T) stretches are commonly located at the ends of yeast small nuclear RNA (snRNA) genes (Parker et al. 1988). A second protected fragment of ~80 nucleotides was also seen in the

lanes with total yeast RNA but not in the lane with the purified 340-nucleotide RNase MRP RNA. This species may originate from either a downstream gene or possibly a tRNA encoded downstream of the *NME1*-coding region, although neither a potential open reading frame nor a tRNA gene was observed for at least 600 bp downstream of the *NME1*-coding region. A 392-nucleotide complementary RNA probe was generated from the region 5' of the *KpnI* site internal to the *NME1* gene. This probe protected 200-, 145-, and 60-nucleotide fragments in all three experimental lanes. The 145- and 60-nucleotide species are likely breakdown products of the 200-nucleotide fragment. Alternatively, they may represent a polymorphism between the yeast strain from which the gene was cloned and the strain from which the RNA was isolated. These 5'- and 3'-end results are consistent with a full-length RNA of 340 nucleotides.

To map precisely the 5'-end nucleotide, a primer extension assay was employed (Fig. 3C; Sambrook et al. 1989). Total yeast RNA was hybridized with a complementary primer (see Materials and methods) and extended with reverse transcriptase. The primer extension products were run in parallel with a DNA sequencing reaction performed with the same oligonucleotide. A

Figure 3. Properties of the *NME1* RNA. (A) Northern analysis of yeast RNA with *NME1*. Total yeast RNA was purified (Schmitt et al. 1990) from cells grown with either 10% or 2% dextrose as a carbon source and analyzed by Northern analysis. Each lane was loaded with 10 µg of total RNA, along with purified RNA from yeast RNase MRP enzyme preparations. The size standards are pBR322 DNA digested with *HpaII*. The blot was probed with a 1.1-kb *EcoRI* fragment encompassing the gene, washed with $0.1\times$ SSPE, 0.1% SDS, at 65°C, and exposed to X-ray film. (B) Analysis of the 5' and 3' ends of the *NME1* RNA using RNA protection assays. The RNA protection assay was performed as described (Sambrook et al. 1989). Total RNA, from yeast grown on 2% dextrose as a carbon source, was analyzed along with purified RNA from yeast RNase MRP enzyme preparations. The probes correspond to T7 polymerase antisense transcripts of the *EcoRI*–*KpnI* and the *KpnI*–*EcoRI* sites shown in Fig. 2A. Five micrograms of *E. coli* tRNA was used as a negative control. Protected products are discussed in the text. The 60-nucleotide species in the RNase MRP RNA (5' end) lane is visible on longer exposure. (C) Primer extension of the *NME1* RNA reveals a single initiation site. A complementary synthetic 21-nucleotide primer corresponding to nucleotides 98–78 of the *NME1* RNA was 5'-end labeled and hybridized to total yeast RNA. The primer was subsequently extended with reverse transcriptase and analyzed on a 6% polyacrylamide/7 M urea gel next to a sequencing reaction utilizing the same primer (Sambrook et al. 1989). The initiating nucleotide corresponds to position +1 in Fig. 2A.

single extension product was observed, which identified the 5' nucleotide as an adenine. This nucleotide is numbered +1 in Figure 2B.

Inhibition of RNase MRP activity with a specific oligonucleotide

To demonstrate further that the RNA purified in our enzyme preparations is an RNA component of the enzyme complex, oligonucleotide inhibition experiments were performed (Fig. 4). The *NME1*-specific oligonucle-

otide, which is complementary to the RNase MRP RNA and gave a single primer extension product (Fig. 3B), inhibits >50% of the enzymatic activity when preincubated with the enzyme at 25 µM and inhibits >75% at 50 µM. An oligonucleotide directed against the 5'-flanking region of the gene gave no inhibition.

NME1 is an essential gene

To examine the phenotype of a yeast cell that has lost RNase MRP, we deleted the *NME1* gene. Using a plas-

Figure 4. Inhibition of RNase MRP activity by an *NME1*-specific oligonucleotide. Purified RNase MRP was incubated either with a specific complementary oligonucleotide (oligo-3, +98 to +78 of the *NME1* gene) or an oligonucleotide directed against the 5'-flanking region (oligo-6, -52 to -32 of the *NME1* gene). Each oligonucleotide was preincubated at 25 μ M or 50 μ M for 3 hr at 4°C. Subsequently, an aliquot of the preincubation, representing 230 μ g of protein, was assayed for RNase MRP activity. After autoradiography, the amount of substrate (147 nucleotides) and product (103 nucleotides) was quantitated for each lane using an AMBIS Radioanalytic Imaging System. After normalizing the amount of product to substrate, the RNase MRP activity was calculated to be inhibited three- and sixfold by 25 μ M and 50 μ M oligo-3, respectively, vs. oligo-6.

mid copy of the *NME1* gene, the coding region from the *HindIII* site to the *NcoI* site was replaced with the yeast *HIS3* gene (Fig. 5A). This removed 299 bp of the 340-bp *NME1* RNA-coding region. After digestion with *EcoRI*, this plasmid was used to transform a *his3*⁻ diploid yeast strain to histidine competence. Because the plasmid does not contain an origin of replication, it must integrate into the chromosome and does so homologously (Rothstein 1991), thus leading to the replacement of the *NME1* gene with the *HIS3* gene.

Histidine-competent yeast strains were tested for proper integration by Southern analysis (Fig. 5B). MES101 is the wild-type diploid strain, and MES106 is the histidine-competent strain. After digestion with *EcoRI*, the wild-type allele *NME1* was seen as a 6.5-kb band, and the deleted allele *nme1-Δ1::HIS3* was seen as a 7.7-kb band. The net 1200-bp increase in size is the result of a loss of 299 bp from the deletion and an increase of 1487 bp from the *HIS3* gene. The difference in band intensity was also expected because the deletion of the *NME1*-coding region results in less DNA available for hybridization to the probe. After digestion with

EcoRI and *PstI*, the wild-type allele migrated as a 2.3-kb band while the deleted allele was seen as two species of 1.65 and 1.8 kb. Two bands were observed in the deleted strain owing to an insertion of a *PstI* site present in the *HIS3* gene. The sum of the two species sizes minus the expected increase in size from the insertion was equivalent to the size of the wild-type allele.

The diploid strain MES106, which contains one wild-type and one deleted copy of *NME1*, was sporulated, and tetrads were dissected (Fig. 5C). Nearly every dissected spore gave rise to two viable spores and two nonviable spores, in a 2 : 0 pattern of segregation. Spores were allowed to grow for >1 week, yet no growth was seen for any of the nonviable spores. After 4 days the nonviable spores were examined and found to contain between one and eight arrested cells. All of the viable spores were found to be *his*⁻. This result is consistent with the *NME1* gene being essential for growth. The strain MES106 (*NME1/nme1-Δ1::HIS3*) was also transformed with the plasmid pMES127, a yeast *CEN URA3* plasmid carrying a complete copy of *NME1*, to create the strain MES108. When sporulated and dissected, MES108 gave rise to spores that were *HIS*⁺, but these spores were also always *URA*⁺. Attempts to select against the *URA3*-containing plasmid with 5-fluoro-orotic acid (Boeke et al. 1987) were unsuccessful under the numerous temperature and carbon source conditions employed. These results are consistent with *NME1* being essential for cell viability and growth. A similar set of experiments was performed deleting the *NME1* gene with the *TRP1* gene, instead of the *HIS3* gene; identical results were obtained (data not shown).

Discussion

Yeast RNase MRP contains a single RNA component

RNase MRP, recently shown to exist in *S. cerevisiae* (Stohl and Clayton 1992), has now been purified several hundredfold. The present isolation scheme yields an enzyme preparation that has one prominent RNA species of 340 nucleotides. We know of no other report of purification of a RNP complex in yeast to yield a single major RNA of complete size. The *S. cerevisiae* nuclear RNase P enzyme has been highly purified, but the RNA associated with it was partially degraded in the process (Lee and Engleke 1989).

Currently, the yeast RNase MRP activity appears specific for mtRNA substrate; we have tried to demonstrate RNase MRP-dependent cleavage on several different potential nuclear RNA substrates but without success (M.E. Schmitt and D.A. Clayton, unpubl.). The size of the yeast RNA component is ~20% larger than its mammalian RNase MRP RNA counterparts, but the yeast RNA is much closer in size than are other yeast snRNAs to their mammalian homologs (Kretzer et al. 1987).

The gene encoding RNase MRP RNA

The sequence of the *NME1* gene matched that of the 340-nucleotide RNA in the RNase MRP enzyme prepa-

Figure 5. The gene *NME1* is essential for viability in *S. cerevisiae*. (A) Construction of the deletion of the chromosomal *NME1* gene. The plasmid pMES132, which contains a 1.1-kb fragment encompassing the gene for *NME1*, was digested with *Hind*III and *Nco*I to remove the coding region for the RNA. The *Hind*III site was blunted and a 1487-bp *Ssp*I–*Bsp*HI fragment containing the *HIS3* gene (Sikorski and Hieter 1989) was ligated in place of the *NME1*-coding region. A linear fragment containing the *HIS3* gene flanked by residual sequences at the 5' and 3' regions of *NME1* was liberated from the plasmid with *Eco*RI and *Xho*I and used to complement the *his3*[−] diploid strain of yeast, MES101, to His⁺. This linear DNA fragment integrates into the yeast chromosome by homologous recombination at the *NME1* 5'- and 3'-flanking regions and replaces the *NME1*-coding region with the *HIS3* gene (Rothstein 1991). (B) Southern analysis of both a wild-type diploid, MES101, and a diploid with one deleted copy of *NME1*, MES106. Five micrograms of genomic DNA from strains MES101 and MES106 was digested with *Eco*RI or *Eco*RI and *Pst*I, separated by electrophoresis on a 0.8% agarose gel, and blotted to nitrocellulose. The filter was then hybridized with a ³²P-labeled probe corresponding to the 1.1-kb *Eco*RI fragment containing *NME1*, washed with 0.1× SSC, 0.1% SDS, at 65°C, and exposed to X-ray film (Sambrook et al. 1989). (C) Tetrad analysis of MES106 showing 2 : 0 segregation. The strain MES106 was plated onto sporulation media for 5 days. Subsequently, spores were dissected by tetrad analysis. All of the viable spores were found to be His[−].

rations and was complementary to a 340-nucleotide RNA in total yeast RNA isolates, confirming that it was a gene expressed *in vivo*. Hybridization of the gene to yeast genomic DNA showed it to be single copy. No other cross-hybridizing species were found on Southern analysis, even with low-stringency washes (data not shown).

The yeast and mouse RNase MRP RNA components are compared in Figure 6. Overall, the yeast and the mouse MRP RNAs are 49% identical, when allowing for gaps. Two regions of more extensive identity are seen,

80% and 74%, which we have termed blocks I and II. The first conserved block contains the GAAAGTC that is found in all RNase MRP RNAs and all RNase P RNAs. The second conserved block contains sequences conserved mainly in RNase MRP RNAs.

Two pseudoknots have been proposed for the RNase MRP RNA structure. The "cage model" contains a 4-bp interaction between the mouse CCCC in block I with the GGGG in block II (Forster and Altman 1990). This pseudoknot is conserved in yeast with a single compensatory base change, CCCC to CTCC and GGGG to

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Figure 6. Sequence identity between yeast and mouse RNase MRP RNA-coding sequences. Homologous regions of the mouse RNase MRP RNA gene (Chang and Clayton 1989) were aligned with the sequence of *NME1*. Identical nucleotides are indicated by vertical lines. The two regions of highest homology are boxed and designated I and II, with the degree of conservation indicated. The overall identity between the yeast and mouse RNAs is 49%. The regions of a proposed pseudoknot are underlined (Topper and Clayton 1990b).

GGAG. A second postulated pseudoknot (Topper and Clayton 1990b) is underlined in Figure 6. This pseudoknot is potentially equivalent to one recently proposed for RNase P (Haas et al. 1991). Although this primary sequence is not conserved between the yeast and the mouse RNase MRP RNAs, compensatory base changes keep the potential base-pairing completely conserved (allowing G : U pairing). It has been suggested that both of these pseudoknots form the core catalytic site of RNase P (Haas et al. 1991), and this may be the case for RNase MRP as well.

Import into mitochondria

This is the first characterized enzyme-associated RNA that would have to be made available to the yeast mitochondrial network to react with its only known substrate. A cytoplasmic lysyl-tRNA has been reported to be imported into yeast mitochondria, although it was found not to be charged, and its function, if any, is unknown (Martin et al. 1979, Tarassov and Entelis 1992). Because yeast have multiple copies of their tRNA genes, genetic analysis to determine factors involved in import of tRNAs into mitochondria would be very difficult. On the other hand, the single-copy yeast RNase MRP RNA gene will provide a powerful model system to study intracellular transport in general and any import of nucleic acids into mitochondria. No other system suitable to both genetic and biochemical manipulation is available

to investigate the potential RNA import machinery. Such studies could lead to eventual strategies for correcting mtRNA defects and deficiencies that have significant pathological consequences (Sato and DiMauro 1991).

Nuclear function of RNase MRP

The gene for the RNA component of RNase MRP was found to be essential for cell viability. This result is consistent with the previously reported nuclear abundance of active enzyme in mammalian cells (Topper and Clayton 1990a,b; Karwan et al. 1991), but does not provide evidence for or against a requirement for maintenance of mtDNA. The extramitochondrial function of the enzyme is likely in the nucleus and still remains to be determined, but is now much more approachable. Two possible nuclear roles that would predict the essential phenotype include rRNA processing and DNA replication. Participation in nuclear DNA replication is attractive, as both the nuclear and mitochondrial functions would then be potentially conserved. In fact, the *NME1* gene cloned here contains a 1-bp mismatch to the cell cycle box seen upstream of the *HO* endonuclease (Nasmyth and Shore 1987). If this element were active, it would turn on the RNase MRP RNA gene exactly when needed at the time of initiation of nuclear DNA replication. A thorough mutational analysis of *NME1* should reveal its major mode of action in the nucleus and provide definitive evidence for any essential role in mtDNA replication.

Materials and methods

Media and strains

Yeast media and genetic manipulations have been described (Sherman et al. 1983). *S. cerevisiae* strains used in this study are listed in Table 1. The *Escherichia coli* strain used for cloning, DH5 α , has the genotype, *psi80dlacZ* Δ M15, *endA1*, *recA1*, *hsdR17* ($r_k^- m_k^+$), *supE44*, *thi-1*, λ^- , *gyrA96*, *relA1*, Δ (*lacZ* *argF*)U169, F^- . *E. coli* strains BB4 and JM107/ λ KC (Elledge et al. 1991) were used for propagation of phage and for recovery of phage DNA inserts, respectively.

Purification of yeast RNase MRP activity

Yeast cells, 161 HS40 (Parikh et al. 1989), were grown in 10 liters of YP-2% dextrose to early saturation phase. Cells were harvested by centrifugation at 4000g for 5 min. Freshly harvested cells (~150 grams) were washed once with 400 ml of double-distilled H₂O and once in breaking buffer B-150 [20 mM Tris-HCl (pH 8.0), 150 mM KCl, 5 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF]. The cells were then resuspended in 150 ml of B-150 buffer and placed in a 400-ml bead beater chamber with 100 grams of glass beads (425- to 600- μ m size). The volume was brought up to 400 ml with B-150 buffer, and the mixture was beaten for five 1-min pulses alternating with 1 min of cooling. The mixture was brought to 500 ml with B-150 buffer and allowed to stir for 5 min. The cells and glass beads were removed by centrifugation twice at 4000g for 5 min. The supernatant, ~350 ml, was centrifuged at

Table 1. Genotypes and sources of strains

Strain	Genotype	Source
161 HS40	<i>MATa ade1 lys1 ρ⁻</i>	R.A. Butow
DBY2006	<i>MATα his3-Δ200 leu2-3,112 ura3-52 trp1-Δ1 ade2-1</i>	D. Botstein
DBY2007	<i>MATa his3-Δ200 leu2-3,112 ura3-52 trp1-Δ1 lys2-801</i>	D. Botstein
MES101	<i>MATa/MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 ADE2/ade2-1 LYS2/lys2-801</i>	this work
MES106	<i>MATa/MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 ADE2/ade2-1 LYS2/lys2-801 NME1/nme1-Δ1::HIS3</i>	this work
MES107	<i>MATa/MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 ADE2/ade2-1 LYS2/lys2-801 NME1/nme1-Δ2::TRP1</i>	this work
MES108	<i>MATa/MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 ADE2/ade2-1 LYS2/lys2-801 NME1/nme1-Δ1::HIS3 [pMES127]</i>	this work
MES109	<i>MATa/MATα his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 ADE2/ade2-1 LYS2/lys2-801 NME1/nme1-Δ2::TRP1 [pMES127]</i>	this work
MES111	<i>MATα his3-Δ200 leu2-3,112 ura3-52 trp1-Δ1 ade2-1 nme1-Δ2::TRP1 [pMES127]</i>	this work
MES120	<i>MATα his3-Δ200 leu2-3,112 ura3-52 trp1-Δ1 ade2-1 nme1-Δ1::HIS3 [pMES127]</i>	this work

100,000g for 100 min. The clarified extract was removed carefully to avoid the loose pellet and the fatty upper layer.

The clarified extract (~240 ml at 20 mg/ml protein) was batch absorbed to 100 ml of DEAE-Sephacel (Pharmacia, Inc.) that had been pre-equilibrated in B-150 buffer. The matrix was subsequently washed three times with 200 ml of B-150 buffer. The enzyme was then eluted from the resin with 100 ml of B-250 buffer (B-150 with 250 mM KCl instead of 150 mM KCl).

The eluent (~100 ml at 1.8 mg/ml of protein) was mixed with 10 grams of Bio-Rex 70 resin (Bio-Rad) that had been pre-equilibrated with B-250 buffer. The mixture was stirred for 15 min and was then poured into a 2.5 × 10-cm column. The column was washed with 10× volumes of B-250 buffer, and the RNase MRP activity was eluted with B-500 buffer (B-150 with 500 mM KCl instead of 150 mM KCl). The protein peak was pooled (~2 ml at 1 mg/ml of protein), concentrated, and desalted to 50 mM KCl on a Centricon-100 (Amicon Corp). Two hundred micrograms of protein from this fraction was then loaded onto a 4-ml 15–30% glycerol gradient in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, and 1 mM PMSF. The gradients were centrifuged for 6 hr at 60,000 rpm in an SW60Ti rotor at 4°C. Pooled peak fractions containing RNase MRP activity (~600 μl at 0.5 mg/ml from one preparation) were pooled and frozen to –70°C. The enzyme remained fully active for only 4–5 weeks but retained some activity for several months. Protein concentrations were determined using a modified Lowry assay with BSA as a standard (Markwell et al. 1978).

Assay of RNase MRP activity

Enzyme assays were performed as described previously (Stohl and Clayton 1992) and utilized the 145-nucleotide *ori5* 4.4 substrate for all experiments.

Labeling and sequencing RNAs

RNAs were 3'-end-labeled with [³²P]pCp, as described previously (Chang and Clayton 1987a), and subjected to electropho-

resis in a 6% acrylamide/7 M urea gel. RNAs were visualized by autoradiography, excised from the gel, and eluted overnight at 37°C in 0.5 M sodium acetate (pH 5.2), 1 mM EDTA, and 0.1% SDS. The RNA was precipitated with ethanol and resuspended in double-distilled H₂O. RNAs were sequenced using the Pharmacia RNA Sequencing Kit (Pharmacia, Inc.).

Cloning and sequencing of NME1

The purified RNase MRP RNA was used to produce a labeled cDNA probe. Labeled cDNA was synthesized with 25 ng of gel-purified RNA using a Prime-It Kit (Stratagene, Inc.). However, Moloney murine leukemia virus (M-MuLV) reverse transcriptase (New England Biolabs) was substituted for T7 DNA polymerase, and the reaction was allowed to proceed for 30 min, incorporating ~2.5 × 10⁶ cpm of [α-³²P]dCTP into cDNA. Using this probe, a yeast (S288C) genomic library (Elledge et al. 1991) in the vector λYES was screened (Sambrook et al. 1989). Two positive clones were identified and plaque purified. Plasmids containing the cloned inserts were obtained using the built-in *cre-lox* recombination system. Two resulting clones, pMES124 and pMES125, were restriction mapped and found to be partially overlapping but different (see Fig. 2). The 1.1-kb *EcoRI* fragment of pMES125 was found to hybridize with the probe and cloned into the *EcoRI* site of pBluescript II KS+ in both orientations to create the plasmids pMES128 and pMES129. A *KpnI* fragment from both of these clones was removed to create the plasmids pMES130 and pMES131. The plasmids pMES128, pMES130, and pMES131 were sequenced with both T3 and T7 primers using the Sequenase Kit, (U.S. Biochemical Corp.). The *KpnI* site was identified from the pMES128 plasmid using the T7 primer.

RNA analysis

Total yeast RNA was prepared as described (Schmitt et al. 1990). Northern analysis was performed as described (Schmitt and Trumpower 1990). Hybridization probes were made using the Prime-It kit (Stratagene, Inc.).

RNA protection assays were performed as described (Sambrook et al. 1989). Antisense RNA probes were synthesized using pMES130 with T7 RNA polymerase (3' probe) and pMES131 with T3 RNA polymerase (5' probe).

Primer extension was performed as described (Sambrook et al. 1989). An oligonucleotide primer, 5'-TTGGTGGGAGACTT-TCAACCC-3', complementary to nucleotides 78–98 of the *NME1* transcript, was used. The sequencing reaction was run with the same primer on the plasmid pMES128 with a Sequenase Kit (U.S. Biochemical Corp.).

Oligonucleotide inhibition assays

Purified RNase MRP was incubated with a complementary oligonucleotide, oligo-3 (5'-TTGGTGGGAGACTTTCAACCC-3'), or a negative control oligonucleotide, oligo-6 (5'-CTAAAT-AGTGGTCTTCACCTG-3'). Oligonucleotides were preincubated at 4°C for 3 hr as described previously (Topper and Clayton 1990a). Samples were then assayed for RNase MRP activity.

Construction of plasmids and yeast strains

The gene for *NME1* was deleted as follows. The plasmid pMES129 was digested with *XhoI* and religated to remove the *HindIII* site in the vector to create the plasmid pMES132. This plasmid contains unique *HindIII* and *NcoI* sites that remove most of the coding region of the gene. The plasmid pMES132 was digested with *HindIII*, blunt-ended, and cleaved with *NcoI*. Either a 1487-bp *BspHI*–*SspI* fragment containing the *HIS3* gene (Sikorski and Hieter 1989) or a 1305-bp *BspHI*–*SspI* fragment containing the *TRP1* gene (Sikorski and Hieter 1989) was ligated into pMES132, replacing the 299-bp fragment internal to the *NME1* gene. This created the plasmids pMES134 (*HIS3* gene) and pMES135 (*TRP1* gene). These plasmids were digested with *EcoRI* and *XhoI* and used to transform the diploid yeast strain MES101 as described (Dunn et al. 1984). MES101 was created by a cross of DBY2006 and DBY2007. Strains containing a properly deleted *NME1* locus were identified using Southern hybridization of genomic DNA (Sambrook et al. 1989). Strains carrying the deletions were named MES106 (*nme1-Δ1::HIS3*) and MES107 (*nme1-Δ2::TRP1*). The plasmid pMES127 was made by placing the 1.1-kb *EcoRI* fragment from pMES128 into the *EcoRI* site of the vector pRS316 (Sikorski and Hieter 1989). This plasmid, when placed into MES106 and MES107 to derive MES108 and MES109, respectively, was used to rescue the lethality of the *NME1* deletion.

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M E Schmitt and D A Clayton

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