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Genetics

The RNA of RNase MRP is required for normal processing of ribosomal RNA

(*Saccharomyces cerevisiae*/*NME1* gene/nucleolus)

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ABSTRACT **We have isolated clones which complement the temperature sensitivity and abnormal rRNA processing pattern of the *rrp2-2* mutant of *Saccharomyces cerevisiae* we previously described. DNA sequencing and restriction analysis demonstrated that all clones contain the *NME1* gene encoding the RNA of the ribonucleoprotein particle RNase MRP. Deletion analysis showed that the *NME1* gene is responsible for the complementation of the *rrp2-2* phenotype. A single base change was identified in the *nme1* gene in the *rrp2* mutant, confirming that the *RRP2* and *NME1* genes are identical. Our experiments therefore indicate that RNase MRP, in addition to its previously reported role in formation of RNA primers for mitochondrial DNA replication [Clayton, D. A. (1991) *Trends Biochem. Sci.* 16, 107–111], is involved in rRNA processing.**

In the yeast *Saccharomyces cerevisiae*, as in most other eukaryotes, all rRNA for cytoplasmic ribosomes, except 5S rRNA, is transcribed as a long precursor transcript (Fig. 1*a*) which is processed into mature rRNA molecules concomitantly with the assembly of ribosomal subunits (1, 2, 4). The most abundant processing intermediates have been identified; based on their structures, a pathway for rRNA processing has been proposed (Fig. 1*b*; refs. 1 and 2).

Even though the major intermediates in rRNA processing were identified more than a decade ago (1, 2), the reactions of the processing pathway are just beginning to be elucidated. Several components of the nucleolus, the subdivision of the nucleus housing rRNA synthesis and most of the rRNA processing and ribosomal assembly reactions, have been shown to play essential roles (reviewed in refs. 5 and 6). The small nucleolar RNA (snoRNA) U3 is necessary for rRNA processing in yeast, frogs, and mammals (7–10), and a requirement for U8 snoRNA has recently been demonstrated in frogs (11). In addition, experiments with *S. cerevisiae* have implicated the snoRNAs U14 (12), snR10 (13), and snR30 (14) as well as the nucleolar proteins NOP1 (15), NSR1 (16–18), and NOP3 and GAR1 (19, 20) in rRNA processing and ribosome formation.

Both our lab (3) and J. Warner's lab (21) have described temperature-sensitive mutants (*rrp2-2* and *rrp2-1*, respectively) which exhibit an altered pattern of rRNA processing intermediates. The most striking feature of these mutants is the accumulation at both permissive and nonpermissive temperatures of two forms of 5.8S rRNA which are extended at the 5′ end relative to the canonical 5.8S molecule (Fig. 1*c*). One form, "5.8S A" rRNA, has 6 or 7 additional bases (3); the other, "5.8S B" rRNA, has 149 additional bases (3, 21). Small amounts of 5.8S A rRNA are found in the wild type, but the 5.8S B rRNA can be detected only in mutant cells. In addition, the mutants exhibit increased accumulation of the primary, 35S transcript as well as a 24S precursor molecule (Fig. 1*c*) found only in small amounts in the wild-type.

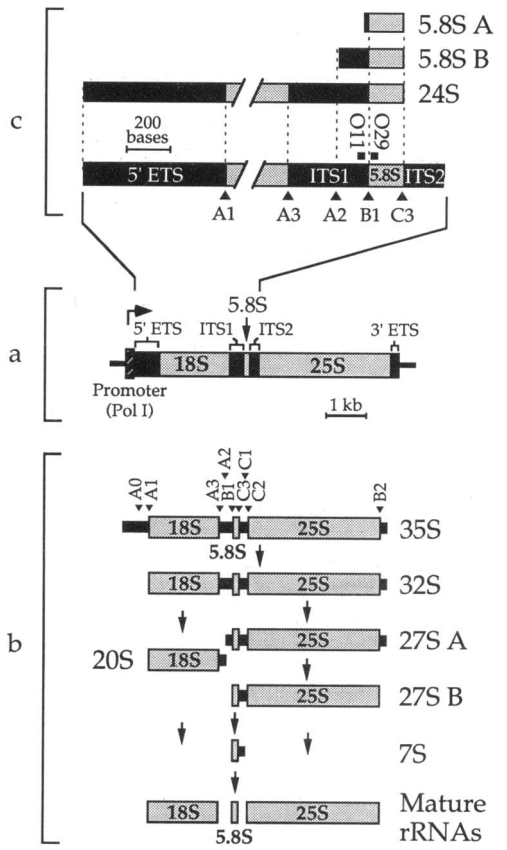

FIG. 1. Pathways for rRNA processing in *S. cerevisiae*. (*a*) Map of the rRNA transcription unit. ETS, external transcribed spacer; ITS, internal transcribed spacer. (*b*) Major processing intermediates in wild-type cells (1, 2). The inferred processing sites are indicated by arrowheads. (*c*) Maps of 5.8S rRNA-containing transcripts characteristic of the *rrp2-2* mutant. Oligonucleotide probes used for Northern analysis are indicated by small black rectangles (see legend to Fig. 2). Proposed processing sites are indicated by arrowheads. Mapping of endpoints of the transcripts in *rrp2-2* was described previously (3).

We have now cloned the *RRP2* gene and shown that it is identical to the gene *NME1* encoding a 339-base RNA molecule which is the *S. cerevisiae* equivalent of the RNase MRP RNA in higher eukaryotes (22). This RNA, also called 7-2 RNA and Th-RNA (23, 24), has been proposed to function in mitochondrial DNA synthesis, because it is part of the ribonucleoprotein RNase MRP (mitochondrial RNA processing) which *in vitro* cleaves RNA molecules corresponding to

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Abbreviation: snoRNA, small nucleolar RNA; ETS, external transcribed spacer; ITS, internal transcribed spacer.

the primer region for mitochondrial DNA replication (25, 26). However, only a minute fraction of the NME1/7-2 RNA is located in the mitochondria (27, 28). Almost all of these RNA molecules are found in the nucleus (27–29), with most or all in the nucleolus (29–31). This localization to the nucleolus has provoked suggestions that the NME1/7-2 RNA may play a role in rRNA processing (5, 25, 31). The results we report here indicate that RNase MRP is indeed involved in rRNA processing.

MATERIALS AND METHODS

Strains and Plasmids. Strains YLL53 (*MATα, ade2-101, his3Δ200, ura3-52, tyr1, RRP2*) and YLL54 [*MATα, ade2-101, his3Δ200, ura3-52, lys2, rrp2-2* (temperature-sensitive)] were derived from sibling spores of a single tetrad obtained in the second backcross of the *rrp2-2* mutant to its parent (3). Strain KS7-1D [*MAT***a**, *ade2, ura3, leu2, trp1, rrp2-1* (temperature-sensitive)] (21) was kindly provided by Jon Warner (Albert Einstein College of Medicine). The *rrp2-1* and *rrp2-2* mutants were isolated independently from different collections of temperature-sensitive strains but were shown to be allelic by virtue of their identical rRNA processing phenotypes and failure to complement each other (3, 21). The YCplac33 cloning vector contains the *CEN4, ARS1*, and *URA3* sequences of *S. cerevisiae* (32) and was obtained from C. H. Sommers and S. Prakash (University of Rochester). DNA manipulations were carried out by standard procedures (33).

Construction of Library and Isolation of Plasmids Complementing the *rrp2-2* Mutation. Genomic DNA was isolated (34) from YLL53 and partially digested with *Sau*3A1 to generate a maximal amount of fragments in the range 5–20 kb. Fragments between 4 and 10 kb were purified by agarose gel electrophoresis and ligated in the presence of YCplac33 DNA which had been digested with *Bam*HI and treated with calf intestinal phosphatase. The plasmid library was then transformed directly into YLL54 (35). Transformed cells were plated on synthetic complete glucose medium (36) lacking uracil and were incubated at 25°C. Temperature-resistant transformants were identified by replica plating to plates incubated at 37°C.

RNA Analysis. Cultures were grown at 25°C in synthetic complete glucose medium with or without uracil as appropriate for maintenance of plasmids. At about 10^7 cells per ml, part of each culture was shifted to 37°C. After an additional 2 hr, total RNA was extracted from both 25°C and 37°C cultures. Aliquots (5 μg) were electrophoresed through urea/polyacrylamide gels and blotted to nylon membranes. Blots were probed with oligonucleotides that were 5′-end-labeled with ^{32}P (3).

Analysis of Genomic Mutations in *rrp2* Mutants. DNA fragments containing the *NME1* gene were prepared from genomic DNA by PCR using primers positioned ≈170 bases upstream and ≈190 bases downstream of *NME1*. PCR fragments were either purified by agarose gel electrophoresis and sequenced directly or cloned into YCplac33 and then sequenced. To assure that the base changes reported reflect the genomic sequences, rather than errors generated during PCR or cloning, we performed at least two sequencing reactions on the products from two PCRs for wild type and *rrp2-2* and four PCRs for *rrp2-1*.

RESULTS

To clone the *RRP2* gene we took advantage of the fact that the *rrp2-2* rRNA processing mutation also results in failure to grow at 37°C. Since we did not find complementing plasmids in the libraries established by Rose *et al.* (37), we constructed a new library by using the *CEN4*-based shuttle vector YCplac33 (32) and wild-type genomic DNA (see *Materials and Methods*). Transformation of this library into an *rrp2-2* mutant strain yielded about 20,000 transformants of which 13 grew at 37°C. Elimination of the plasmids from these 13 strains with 5-flouroorotic acid (38) resulted in loss of the ability to grow at 37°C, demonstrating that the temperature resistance was due to the plasmids in these strains, rather than reversion or genomic suppressor mutations. Plasmid DNAs from five of the temperature-resistant strains were amplified in *Escherichia coli* and used for retransformation of the *rrp2-2* mutant. Northern analysis of RNA from the transformants showed the wild-type pattern of 5.8S rRNA molecules—i.e., little or no accumulation of 5.8S A and 5.8S B (Fig. 2 and data not shown). Thus, the plasmids complement the abnormal rRNA processing phenotype in the *rrp2-2* mutant.

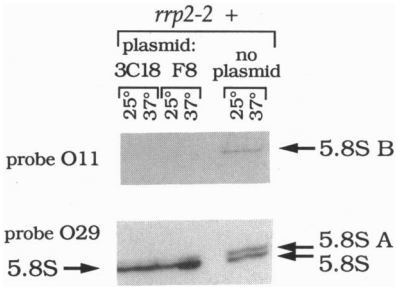

FIG. 2. Northern analysis of 5.8S rRNAs from strains carrying plasmids complementing *rrp2-2* temperature sensitivity. Total RNA was prepared from mutant strains carrying complementing plasmids 3C18 or F8 (see Fig. 3). RNA was also prepared from *rrp2-2* cells without plasmid. Oligonucleotide probe O11 is complementary to ITS1, 13–37 nucleotides upstream of the 5′ end of canonical 5.8S rRNA, and hence detects 5.8S B rRNA but not 5.8S or 5.8S A rRNAs (Fig. 1*c*). Probe O29 is complementary to nucleotides 24–48 of canonical 5.8S rRNA (Fig. 1*c*).

Analysis of the inserts in the five clones by restriction enzyme digestion revealed that two of the five clones were identical and that all inserts represented overlapping fragments of the same region of the genome (Fig. 3). We sequenced portions of several complementing plasmids and searched the GenBank data base (Release 75.0) (39) for matches by using the BLAST network service at the National Center for Biotechnology Information. This search revealed that the *rrp2-2*-complementing plasmids carried the *NME1* gene previously cloned by Schmitt and Clayton (22).

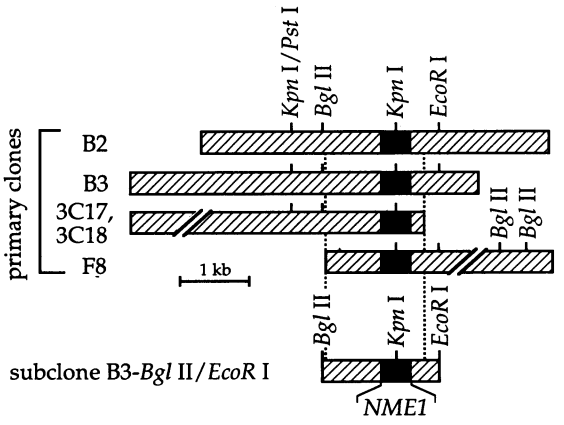

FIG. 3. Maps of cloned fragments on plasmids complementing *rrp2-2* temperature sensitivity. Pertinent restriction enzyme recognition sites are shown. The *NME1* gene (22) is indicated by the solid black rectangle. The region between the dotted lines indicates the minimal DNA sequence common to all complementing plasmids.

Because *NME1* transcripts (and the 7-2 RNA equivalent in mammals and plants) have previously been localized to the nucleolus (30, 31) and because ribonucleoprotein particles containing these RNA molecules have RNase activity (25), we suspected that the complementation of defective rRNA processing in the *rrp2-2* mutant was due to the *NME1* gene itself, rather than a closely linked gene which might also be present within the inserts of our complementing plasmids. To test this, we first constructed a subclone which carried only ≈1.6 kb of DNA (Fig. 3) but still complemented both temperature sensitivity of growth and abnormal processing of 5.8S rRNA (data not shown). We then used BAL-31 exonuclease to generate short deletions around the *Kpn* I site within the *NME1* gene on the subclone (Fig. 4). These deletions were tested for their ability to complement growth at 37°C of the *rrp2-2* mutant (Fig. 5). Plasmids with internal deletions of ≥22 bp failed to support growth at 37°C, consistent with our hypothesis that *NME1* complements the *rrp2-2* mutation. Interestingly, smaller deletions removing 14 nucleotides or less of the *NME1* RNA had little or no effect on the ability of the plasmid to restore growth of the *rrp2-2* mutant strain at 37°C (Fig. 5). Processing of rRNA in strains carrying the 14-, 22-, and 65-base deletions was tested by Northern analysis of the 5.8 S rRNAs (Fig. 6). As expected, the 14-base deletion had no effect on the ability of the plasmid-borne *NME1* gene to complement the rRNA processing defect, but the longer deletions eliminated the ability to complement. Thus, the deletion analysis confirmed our hypothesis that complementation of the *rrp2-2* mutation is due to the *NME1* gene.

To confirm that the *NME1* and *RRP2* loci are identical, we sequenced the *NME1* gene from wild type as well as the *rrp2-1* and *rrp2-2* mutants, using DNA fragments generated by PCR amplification of genomic DNA. If the two genes are identical the sequence of the chromosomal copy of *NME1* in the *rrp2* mutant should differ from the *NME1* gene in the wild-type sibling. On the other hand, if the complementation is due to extragenic suppression by the cloned *NME1* gene, the chromosomal *NME1* gene in the *rrp2* mutants should be wild type. Indeed, we found a G-to-A substitution at nucleotide 122 of the *NME1* gene in both *rrp2* mutants (Fig. 4).

To gain a preliminary understanding of the nature of the processing defect in the *rrp2-2* and deletion mutants, we asked whether the mutant *NME1* RNA transcripts were unstable and hence accumulated to a reduced level. Northern blots of RNA from wild type and the *rrp2-2* mutant showed no detectable difference in the concentration of the *NME1* transcripts in the two strains (Fig. 7*a*). Thus, the processing defect in the *rrp2-2* mutant is most likely due to a defect in the catalytic activity of the RNA–protein particle, rather than a reduced concentration. In contrast, the failure of the 22- and 65-base deletions to complement may be due to instability of the transcript, since only small amounts of these transcripts could be detected (Fig. 7*b*), whereas the complementing 14-base deletion transcript accumulated to the same level as

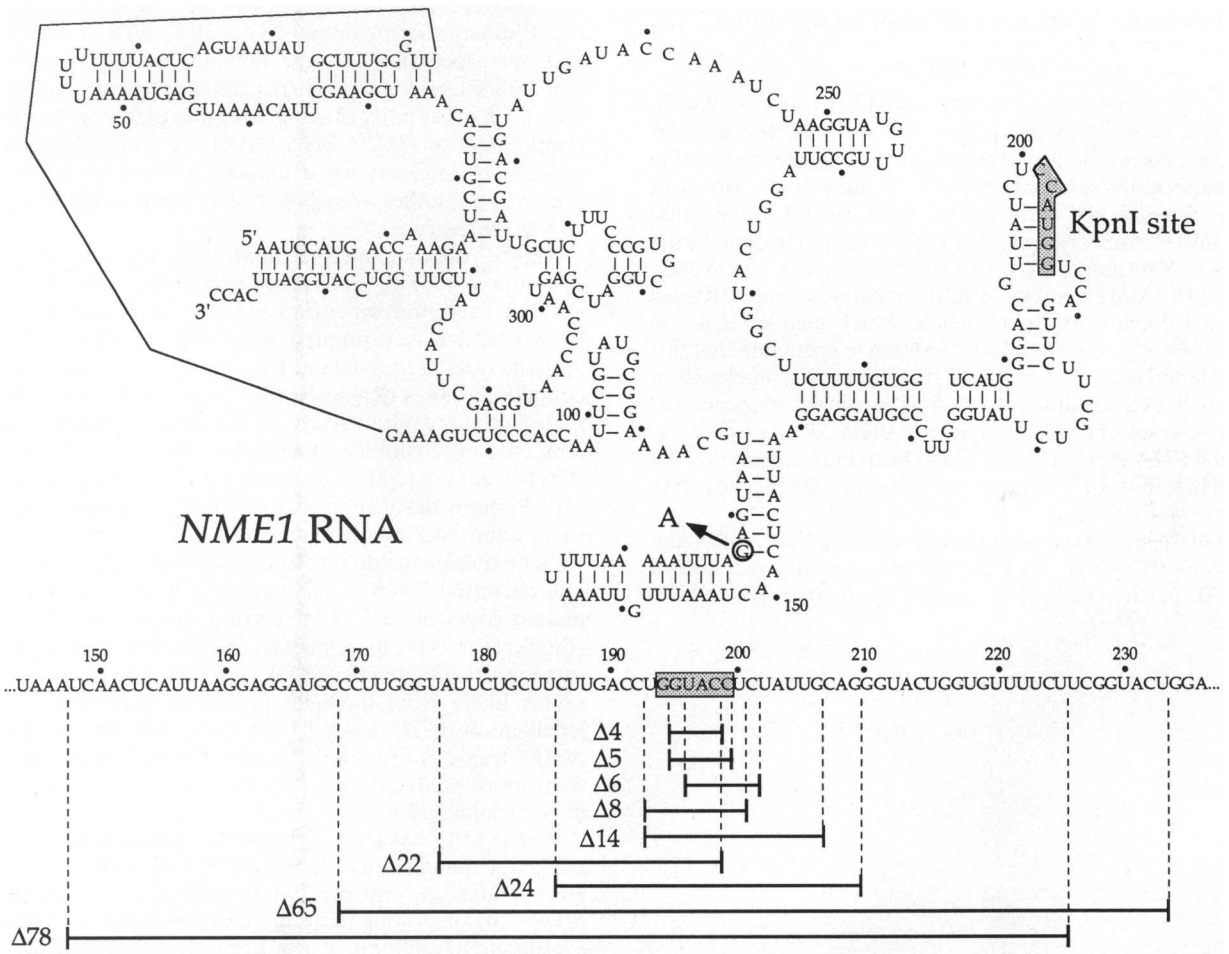

FIG. 4. Structure of the *NME1* RNA of *S. cerevisiae*. The secondary structure of the *NME1* RNA, proposed by Schmitt *et al.* (40), is shown at the top. The recognition sequence for *Kpn* I is indicated. Below is a linear representation of the RNA sequence flanking the *Kpn* I site. The various deletion derivatives of subclone B3-*Bgl* II/*Eco*RI (Fig. 3), generated by BAL-31 treatment of *Kpn* I-linearized DNA, are shown. The base substitution at nucleotide 122 found in both *rrp2* mutants is indicated.

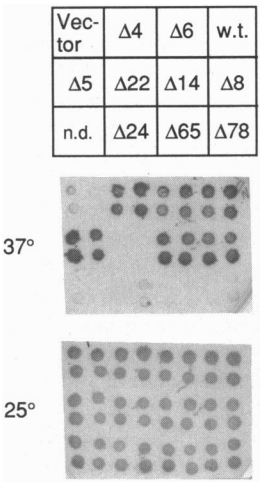

FIG. 5. Complementation of *rrp2-2* temperature sensitivity with deletion derivatives of *NME1*. Strain *rrp2-2* was transformed with the B3-*Bgl* II/*Eco*RI subclone (Fig. 3) or derivatives of this with internal deletions in the *NME1* gene (Fig. 4). Two colonies from each transformation were spotted in duplicate on synthetic complete glucose medium lacking uracil and then were incubated at 25°C. After the patches grew up they were replicated to two new plates; one plate was incubated at 37°C and the other at 25°C for 2–3 days. A long deletion whose endpoints were not determined is indicated (n.d.). w.t., Wild type.

the wild-type and *rrp2-2* transcripts (visible as a doublet in a shorter exposure of the autoradiogram shown in Fig. 7*b*).

DISCUSSION

The results demonstrate that normal rRNA processing in *S. cerevisiae* requires the RNA product of the *NME1* gene. A single base change in this gene results in abnormal processing and temperature-sensitive growth. In agreement with our results, Schmitt and Clayton (44) have recently found that repression of transcription of *NME1* results in accumulation of 5.8S rRNA molecules with extended 5′ ends. The *NME1* transcript (and its equivalent in mammals) is part of RNase MRP, a ribonucleoprotein particle which cleaves RNA *in vitro* (25, 26, 41). It is therefore tempting to speculate that this ribonucleoprotein particle also functions as a nuclease in rRNA processing, although there is no direct evidence for this. A number of other ribonucleoprotein particles containing small RNA molecules have also been implicated in rRNA processing, but so far none of these has been shown to function as a nuclease.

Although our experiments clearly implicate *NME1* RNA in processing of rRNA, they do not pinpoint the reaction in which it participates. One possibility is that the *NME1*

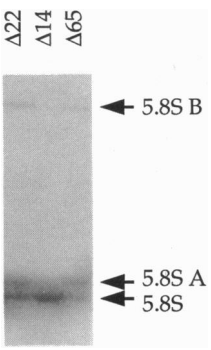

FIG. 6. Northern analysis of *rrp2-2* strains carrying plasmids with 14-, 22-, and 65-base deletions in the *NME1* gene. The blot was probed with oligonucleotide O29 (Fig. 1).

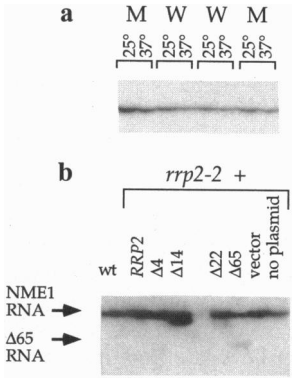

FIG. 7. Northern analysis of accumulation of *NME1* transcripts. (*a*) RNA prepared from the offspring of the four spores in a tetrad from the backcross of *rrp2-2* to its wild-type parent. W, wild type; M, *rrp2-2* mutant. (*b*) RNA prepared from 37°C cultures of wild-type (wt) cells and of *rrp2-2* cells with or without plasmids carrying the indicated derivatives of *NME1*. The blots were probed with an oligonucleotide complementary to bases 299–319 of the *NME1* transcript (Fig. 4).

transcript is involved in the formation of the 5′ end of the canonical 5.8S rRNA, since the *rrp2* mutants accumulate large amounts of 5.8S rRNA with extended 5′ ends (3, 21). However, the *rrp2* mutations also affect processing in the 5′ ETS, as evidenced by the increased accumulation of 35S and 24S transcripts and concomitant reduction in 32S transcripts (3, 21). We cannot distinguish whether the *NME1* transcript has a direct role in both ITS1 and 5′ ETS processing or whether processing of these domains is interdependent so that interference with one processing reaction has indirect effects on other parts of the processing pathway. In fact, the requirement of *NME1* RNA for rRNA processing could be even more indirect; for example, it might be involved in processing another snoRNA which participates directly in rRNA processing.

Another outstanding issue is why the *rrp2* mutants grow at 25°C but not 37°C, yet rRNA processing appears equally abnormal at both temperatures (3, 21). Both 5.8S A and 5.8S B rRNAs are incorporated into 60S subunits in the *rrp2* mutants (ref. 21 and data not shown). Even though subunits containing 5.8S A rRNA are also found in wild-type cells (42), their increased abundance in the mutant might result in temperature sensitivity. In addition, subunits containing 5.8S B rRNA appear to enter polysomes very inefficiently at 23°C (21). Perhaps these abnormal subunits block protein synthesis at nonpermissive temperature.

Our experiments do not address the role of RNase MRP in mitochondrial DNA replication. We know that the *rrp2-2* mutant grows at 25°C on glycerol, a nonfermentable carbon source. However, there may be enough RNase MRP activity to support mitochondrial DNA replication. In any case, it seems likely from the data presented here and from the localization of 7-2 RNA to the nucleolus that most of the *NME1* transcript functions in rRNA processing. Therefore, we propose calling the gene *RRP2*, since this refers to its role in ribosomal RNA processing.

RNase MRP has an intriguing relationship to RNase P, the enzyme which creates the mature 5′ end of tRNAs. Certain autoimmune antisera precipitate both RNase P and RNase MRP (23), suggesting that they share an antigen, most likely a protein. In addition, it has been proposed that the RNA components of RNase MRP and RNase P have similar secondary structures (40, 43). It will be interesting to learn whether there are any important functional similarities between RNase MRP and RNase P.

We thank Chris Sommers for advice on construction of the library and Daryia Vorozheykina for helpful suggestions on sequencing of PCR-generated DNA fragments. We thank the National Center for Biotechnology Information for their services. This work was supported by a grant from the National Institute of Allergy and Infectious Diseases.

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