THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 12, Issue of March 25, pp. 11352–11360, 2005
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Characterization and Purification of *Saccharomyces cerevisiae* RNase MRP Reveals a New Unique Protein Component*

Received for publication, August 19, 2004, and in revised form, December 22, 2004
Published, JBC Papers in Press, January 6, 2005, DOI 10.1074/jbc.M409568200

Kelly Salinas‡, Sara Wierzbicki‡, Li Zhou, and Mark E. Schmitt§

From the Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, Syracuse, New York 13210

In the yeast *Saccharomyces cerevisiae*, RNase mitochondrial RNA processing (MRP) is an essential endoribonuclease that consists of one RNA component and at least nine protein components. Characterization of the complex is complicated by the fact that eight of the known protein components are shared with a related endoribonuclease, RNase P. To fully characterize the RNase MRP complex, we purified it to apparent homogeneity in a highly active state using tandem affinity purification. In addition to the nine known protein components, both Rpr2 and a protein encoded by the essential gene *YLR145w* were present in our preparations of RNase MRP. Precipitation of a tagged version of Ylr145w brought with it the RNase MRP RNA, but not the RNase P RNA. A temperature-sensitive *ylr145w* mutant was generated and found to exhibit a rRNA processing defect identical to that seen in other RNase MRP mutants, whereas no defect in tRNA processing was observed. Homologues of the Ylr145w protein were found in most yeasts, fungi, and *Arabidopsis*. Based on this evidence, we propose that *YLR145w* encodes a novel protein component of RNase MRP, but not RNase P. We recommend that this gene be designated *RMP1*, for RNase MRP protein 1.

RNase MRP[1] is a highly conserved and essential ribonucleoprotein endoribonuclease that cleaves substrates in at least two intracellular compartments. Most RNase MRP is localized to the nucleolus (1), where a role in processing of rRNA precursors has been identified (2). RNase MRP-mediated cleavage at the A_3 site of pre-rRNA ultimately leads to the generation of 5.8S(S) rRNA (3–5). A fraction of RNase MRP RNA finds its way to the mitochondrion (6, 7). Mitochondrial RNase MRP processes RNA transcripts, which serve as primers for the leading strand of mitochondrial DNA replication (8, 9). Recent data also support a role for RNase MRP mRNA degradation, whereby cleavage of the 5′-untranslated region (UTR) of *CLB2* mRNA, which encodes a B-type cyclin, leads to its rapid degradation and aids cell cycle progression (10).

In *Saccharomyces cerevisiae,* all the known components of RNase MRP are essential for viability. To date, a single RNA component and at least nine protein components of nuclear RNase MRP have been identified. The subunit composition of RNase MRP closely resembles that of a related ribonucleoprotein endoribonuclease, RNase P, which processes tRNA precursors to generate mature 5′ termini. Eight of the proteins associated with RNase MRP (Pop1, Pop3, Pop4, Pop5, Pop6, Pop7, Pop8, and Rpp1) are also components of RNase P (11–14). An RNA-binding protein, encoded by the gene *SNM1*, is the only known protein component that associates with RNase MRP RNA but not RNase P RNA (15). Similarly, Rpr2p has been identified as a unique protein component of the RNase P complex (14).

The similarities between RNase MRP and RNase P extend beyond that of shared protein components. The RNA subunits of RNase MRP and RNase P are evolutionarily and structurally related (16, 17). They share only weak sequence homology, but they fold into similar cage-like secondary structures (16). In addition to subunit composition, both RNase MRP and RNase P localize to the nucleolus and the mitochondria and have been shown to cleave common substrates (14, 18).

Despite their similarities, RNase MRP and RNase P appear to assemble into separate catalytic complexes. Nuclear RNase P, purified to homogeneity by high-resolution anion exchange chromatography, retains tRNA processing activity independently of RNase MRP (5, 14). In this study, we outline a method for purifying nuclear RNase MRP in *S. cerevisiae* to apparent homogeneity using a tandem affinity purification system (19). Characterization of the purified complex confirms that the MRP RNA and nine previously identified proteins are components of the RNase MRP complex. In addition, we found the protein encoded by the essential gene *YLR145w* and the gene *RPR2* in preparations of purified and active RNase MRP. We demonstrate that the Ylr145w protein is indeed a protein component of RNase MRP essential for its activity but is not a component of RNase P. This will be the second unique protein component of RNase MRP. We recommend the new name for this gene be designated *RMP1*, for RNase MRP protein 1.

MATERIALS AND METHODS

Strains and Media—Yeast media and genetic manipulations have been described previously (10, 20). The *Escherichia coli* strain used for cloning, DH5α, has the genotype *ϕ80dlacZΔM15 endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ^- *gyrA96 relA1 Δ(lacIZYA-argF)U169* F^-. Basic molecular techniques were performed as described previously (21). To purify RNase MRP, the YSW1 strain, which has the genotype *MAT***a** *POP4*::*TAPTAG*::*TRP1ks pep4*:*LEU2 nuc1*::*LEU2 sep1*::*URA3 trp-1his3–11,15 can-100 ura3–1 leu2–3,112*, was used (10). This strain was constructed by using PCR to amplify the TAP fusion cassette from pBS1479 (a gift from Bertrand Séraphin) and then using PCR-based genomic TAP tagging to integrate the tag into the carboxyl-terminal codon of the POP4 gene (19). LSY389–34A, which has the genotype *MAT***a** *sep 1*::*URA3 pep4*::*LEU2 nuc1*::*LEU2 ade2–1 trp1–1 his3–11,15*

* This work was supported by National Institutes of Health Grant GM064634 from the NIGMS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, 750 E. Adams St., Syracuse, NY 13210. Tel.: 315-464-8713; Fax: 315-464-8750; E-mail: schmittm@upstate.edu.

[1] The abbreviations used are: MRP, mitochondrial RNA processing; UTR, untranslated region; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TAP, tandem affinity purification; IgG-POD, peroxidase-conjugated antibody.

This paper is available on line at http://www.jbc.org

can1–100 ura3–1 leu2–3,112, was used as the wild-type control strain in PCR, Western blot, and immunoprecipitation analysis. The heterozygous *YLR145w* yeast knock-out strain KLS115 (*MAT*a/∝ *his3-Δ1/his3-Δ1 leu2-Δ0/leu2-Δ0 ura3-Δ0/ura3-Δ0 met15-Δ0/MET15 LYS2/lys2-Δ0 YLR145w/YLR145w*::*KanMX4*) and the TAP-tagged *YLR145w* strain KLS116 (*MAT***a** *YLR145w*::*TAPTAG*::*HIS3 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were obtained from Open Biosystems (Huntsville, AL) unless noted yeast were grown on YPD (1% yeast extract, 2% peptone, 2% dextrose).

Whole-cell Western Blot Analysis—Whole-cell yeast protein extracts were prepared as described previously (20). Samples were denatured in 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol for 5 min at 95 °C and resolved on a 12% polyacrylamide gel (21). Separated proteins were electrophoretically transferred to a BA-S NC-supported nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) by use of a Bio-Rad semi-dry transfer cell. The membrane was stained for 4 min with Ponceau S solution (0.1% Ponceau S, 5% acetic acid) and washed with double distilled H_2O to ensure proper transfer. The membrane was blocked with 5% (w/v) milk-TBST (20 mM Tris/Cl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) for 1 h at 24 °C, incubated with primary antibody for 2 h, and washed with TBST three times for 10 min each. The washed blot was then incubated with a peroxidase-conjugated secondary antibody (IgG-POD) for 1 h and washed with TBST four times for 15 min each. The IgG-POD was detected with a Roche Applied Science chemiluminescence Western blotting kit and exposed to film for 30 min and then exposed to film again overnight. Goat anti-protein A antibody was used at a 1:500 dilution (Polysciences, Warrington, PA). Peroxidase-conjugated anti-goat antibody was used at 1:1000 dilution (Roche Applied Science).

Precipitation of TAP-tagged Protein—Forty μl of rabbit IgG agarose (Sigma), which was equilibrated in ice-cold buffer A (20 mM Tris/Cl (pH 8.0), 150 mM KCl, 5 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), was incubated with 40 μl of whole-cell yeast extract on ice for 1 h. The samples were washed three times with 0.5 ml of buffer A. The volume of each sample was brought up to 100 μl with buffer A. The samples were made 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and heated at 95 °C for 5 min. After the beads were removed by centrifugation at 16,000 × *g* for 5 min, the RNA was extracted with phenol/chloroform equilibrated to pH 5.3, precipitated with ethanol, and examined by Northern analysis.

Purification of Nuclear RNase MRP Using Tandem Affinity Purification—RNase MRP was purified from strain YSW1 as described in detail previously (10). Several individual preparations were pooled for further analysis.

Western Blot Analysis of RNase MRP Protein Components—For each antibody tested, 2 μg of purified nuclear RNase MRP was denatured as described above, resolved on a 15% polyacrylamide gel, and transferred to a BA-S NC-supported nitrocellulose membrane (Schleicher & Schuell, Inc.). Each membrane was blocked in 5% (w/v) milk-phosphate-buffered saline (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4) and 1 mM EDTA for 1 h at room temperature; incubated with a primary antibody for 2 h; washed with 5% (w/v) milk-phosphate-buffered saline, 0.1% (v/v) Triton X-100, 0.02% (w/v) SDS, and 1 mM EDTA for one quick wash and three 10-min washes; blotted with an anti-rabbit IgG-POD (1:1000 dilution; Roche Applied Science) for 1 h; and washed with 10 mM $NaPO_4$ (pH 7.4), 0.5 M NaCl, 0.1% Triton X-100, and 0.02% SDS for one quick wash followed by four washes for 15 min each. The anti-rabbit IgG-POD was detected with a Roche Applied Science chemiluminescence Western blotting kit and exposed to film for 10 s, 30 s, and 5 min. The following primary rabbit antibodies were used: anti-Snm1 (1:2500; Ref. 15); anti-Pop1 (1:1000; Ref. 22); anti-Pop3 (1:500; Ref. 22); anti-Pop4 (1:1000; Ref. 22); and anti-Rpp1 (1:2000; Ref. 22).

MALDI-TOF Mass Spectrometry Analysis of Purified Nuclear RNase MRP—Three hundred μg of purified RNase MRP was run on a 15% SDS-PAGE gel and stained with SYPRO Ruby (Bio-Rad). Individual protein components were excised from the gel using a fresh razor blade. Each gel slice was cut into 1 × 1-mm cubes and placed in a 0.65-ml siliconized microcentrifuge tube (PGC Scientific, Frederick, MD) The gel pieces were washed with double distilled H_2O and then destained twice at 37 °C with 100 mM ammonium bicarbonate and 50% (v/v) acetonitrile. The gel pieces were dehydrated in 100% acetonitrile and dried under vacuum. The gel pieces were then reduced at 56 °C for 30 min in 10 mM dithiothreitol/100 mM ammonium bicarbonate. The pieces were dehydrated with 100% acetonitrile and then alkylated by 55 mM iodoacetamide/100 mM ammonium bicarbonate for 30 min in the dark at room temperature. After washing with 100 mM ammonium bicarbonate, the gel pieces were dehydrated in 100% acetonitrile and dried under vacuum. The pieces were re-swollen in 50 mM ammonium bicarbonate containing sequencing-grade modified trypsin (Promega) at a final concentration of 12.5 ng/ml and placed on ice for 1 h. Excess buffer was removed, and then the gel pieces were resuspended in 50 mM ammonium bicarbonate. The gel pieces were digested overnight at 37 °C.

The digested peptides were mixed vigorously for 10 min and extracted from the gel pieces with double distilled H_2O. The digested products were extracted twice for 1 h with 50% (v/v) acetonitrile/5% (v/v) formic acid. The extracts were pooled together, spun at 16,000 × *g* for 5 min to remove contaminating gel pieces, and then dried under a vacuum. Each sample was resuspended in 8 ml of 0.1% trifluoroacetic acid and passed through a C18 microzip tip (Millipore, Bedford, MA) before MALDI-TOF mass spectrometry analysis.

Mass spectrometry data were collected at the Proteomics Core Facility at State University of New York Upstate Medical University using a TOF Spec 2E mass spectrometer (Waters Corp., Beverly, MA.) All samples were analyzed under identical parameters in reflectron mode. The protein mass spectra generated were analyzed using Pro Found (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

PCR Mutagenesis of YLR145w—Plasmid pKLS108 (*URA3 CEN YLR145w*) was used as a template for PCR mutagenesis (23). The oligonucleotides YLR145wFOR (5′-GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTCACCTGCCACCCTATTTTC-3′) and YLR145wREV (5′-CCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTACTTGGGCAGACAAGGTC-3′) were used to amplify a 1.1-kb gene product that contained the *YLR145w* gene. Error-prone Deep Vent (exo-) polymerase (New England Biolabs) was used to increase the mutation rate.

The plasmid YCplac111 (*LEU2 CEN*) contains unique HindIII and BamHI restriction sites that were used to remove a 30-bp region surrounding the multiple cloning site (24). Purified mutagenized *YLR145w* PCR product and the gapped YCplac111 plasmid were co-transformed into the haploid strain KLS111 (*MATα his3-Δ1 leu2-Δ0 ura3-Δ0 ylr145w*::*KanMX4 pKLS108* [*URA3 CEN YLR145w*]) to achieve *in vivo* gap repair of YCplac111. Leu^+ transformants were transferred to plates containing 0.2% (w/v) 5-fluoroorotic acid (25). Transformants were passed a second time on 5-fluoroorotic acid medium to ensure that the *URA3*-containing plasmid was lost. Mutants that exhibited a temperature-conditional growth phenotype were selected for further analysis. The YCplac111 plasmid was recovered from the mutant yeast strain via transformation of *E. coli* with yeast cell lysates (20). The isolated plasmid was re-transformed into KLS111 to ensure that the mutations were linked to the plasmid. The entire *YLR145w* gene was sequenced using M13 forward and reverse primers to identify mutations.

Analysis of Yeast RNA—Analysis of yeast RNA by Northern analysis was performed as described previously (26, 27). Probes used for Northern analysis were RNase MRP RNA (1,088-bp EcoRI fragment of pMES140 (*LEU2 CEN NME1*) encompassing the *NME1* gene (28), RNase P RNA (450-bp fragment stretching from −22 to +428 of the *RPR1* gene), and $tRNA_{Arg}$ (178-bp fragment from −71 to +107 of the *tR(ACG)L* gene). Probes were radiolabeled for hybridization with [α-^{32}P]dCTP using the Prime-It Kit (Stratagene, La Jolla, CA). Radioactive blots were analyzed on an Amersham Biosciences PhosphorImager.

RESULTS

The RNase MRP Complex Was Purified using TAP-tagged Pop4—Purification of RNase MRP has been recalcitrant to standard techniques due to sensitivity of the RNA component of the enzyme to contaminating RNases and to low yields. Previous research aimed at characterization of the complex has relied upon fractionation techniques to purify RNase MRP-containing extracts or mutational and/or genetic analyses of known RNA or protein components (2, 14, 15, 28). We have generated a yeast strain (YSW1) with a modified *POP4* locus from which RNase MRP can be purified to homogeneity. To alleviate problems associated with nuclease digestion, the strain contains deletions in two of the major cellular nucleases, *NUC1* and *XRN1* (29). To simplify the purification and to optimize yields, we utilized a TAP methodology (19), with minor modifications. The cassette consists of a calmodulin-binding domain, a TEV protease cleavage site, the IGG-binding domain of protein A from *Staphylococcus aureus*, and a

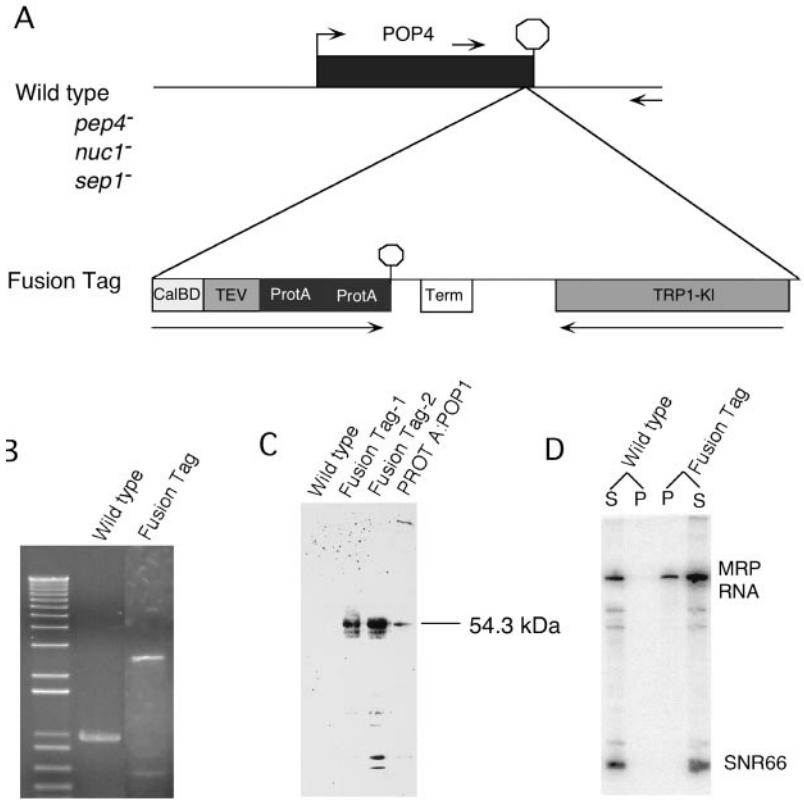

FIG. 1. **Construction of a yeast strain expressing TAP-tagged Pop4.** *A*, targeting of the TAP fusion cassette to the *POP4* locus. The TAP fusion tag, which consists of protein A from *S. aureus*, a TEV protease cleavage site, a calmodulin-binding domain, and a *TRP1* marker, was amplified by PCR from pBS1479 and targeted to the carboxyl terminus of the *POP4* locus (19). *B*, PCR analysis of the *POP4* locus. Genomic DNA from a wild-type strain and the transformed Pop4 fusion strain was used to amplify the *POP4* locus by PCR. Products were run on an agarose gel and stained with ethidium bromide. The fusion-tagged strain exhibits a 1700-bp shift, consistent with the size of the TAP cassette. *C*, Western blot analysis of the Pop4 fusion strain. Whole-cell protein extracts were run on 12% SDS-PAGE and transferred to nitrocellulose. The blot was incubated with anti-protein A antibody and then probed with a secondary peroxidase-conjugated anti-goat antibody and visualized with the Roche Applied Science chemiluminescence kit. Pop1 tagged with protein A was used as a positive control. *D*, co-immunoprecipitation of MRP RNA from the Pop4 fusion strain. Whole-cell extracts were incubated with IgG beads for 60 min and washed several times. RNA was extracted from bound (*P*) and unbound (*S*) fractions using phenol/chloroform and precipitated. RNA was run on a 6% acrylamide-7 M urea gel, transferred to nylon, and detected by hybridization with a ^{32}P-labeled probe corresponding to the 1088-bp EcoRI fragment containing the *NME1* and *SNR66* genes.

yeast *TRP1* marker. This cassette was integrated into the carboxyl-terminal codon of the *POP4* gene at its chromosomal locus (Fig. 1*A*). PCR amplification of the *POP4* gene in the resulting YSW1 strain was done to confirm proper integration of the TAP cassette (Fig. 1*B*). YSW1 grew at rates comparable to wild type at all temperatures tested and maintained normal rRNA processing. The modified Pop4 was examined in whole-cell extracts to ensure that it was not rapidly degraded with addition of the tag or that the tag was being rapidly removed (Fig. 1*C*). In the YSW1 strain, a 54.3-kDa band is clearly visible, which corresponds to the expected size of the Pop4-TAP tag fusion protein. Immunoprecipitation of the Pop4 tag using rabbit IgG was also performed to confirm association of the modified Pop4 with the MRP RNA. As shown in the Northern analysis of the co-immunoprecipitation experiment (Fig. 1*D*), the wild-type control strain does not precipitate MRP RNA. However, the TAP fusion strain precipitated MRP RNA, indicating that the TAP tag effectively isolates RNA molecules associated with Pop4.

After confirming that the modified Pop4 protein was functioning comparable to the wild-type protein, we were able to purify the nuclear RNase MRP complex using tandem affinity purification. Because the Pop4 protein is also a component of the RNase P complex, we modified the standard purification procedure to maximize yields of RNase MRP while minimizing co-purification of RNase P. This was accomplished mainly by breaking and extracting cells in a low to moderate salt concentration. RNase MRP was readily released under these conditions, whereas a majority of the RNase P was retained in the cells. In addition, the RNase P complex was found to bind poorly to the calmodulin beads in the final stage of the purification. Samples were collected throughout the purification process, and both total RNA and protein were analyzed (Fig. 2). In the protein A elution, small amounts of the RNase P RNA were detected; however, this RNA was $<1\%$ of the total RNA after the final calmodulin step, as measured by fluorescence intensity of the ethidium bromide-stained RNA. The final elution from the calmodulin beads resulted in a single band consistent in size with *NME1*, the 340-nucleotide component of RNase MRP (Fig. 2*A*). The identity of this band was confirmed by Northern analysis (Fig. 2*B*). Indeed, very little breakdown product of the MRP RNA was detected, indicating the complex is whole. The enzyme complex was found to be highly active on both the rRNA A3 substrate and *CLB2*-5′-UTR substrate, indicating that all of the components required for RNase MRP function are present (10).

The complex was analyzed on a 15–30% glycerol gradient (37) to ensure it was a single complex. As can be seen in Fig. 3*A*, a single peak was present that contained all of the RNase MRP proteins. Spreading of a trace amount of the Pop4 protein and the Rpp1 protein indicates that a very small amount of a partial complex of these two proteins may be co-purifying with the complex. This may represent an intermediate during RNase MRP and RNase P assembly or may simply be a break-

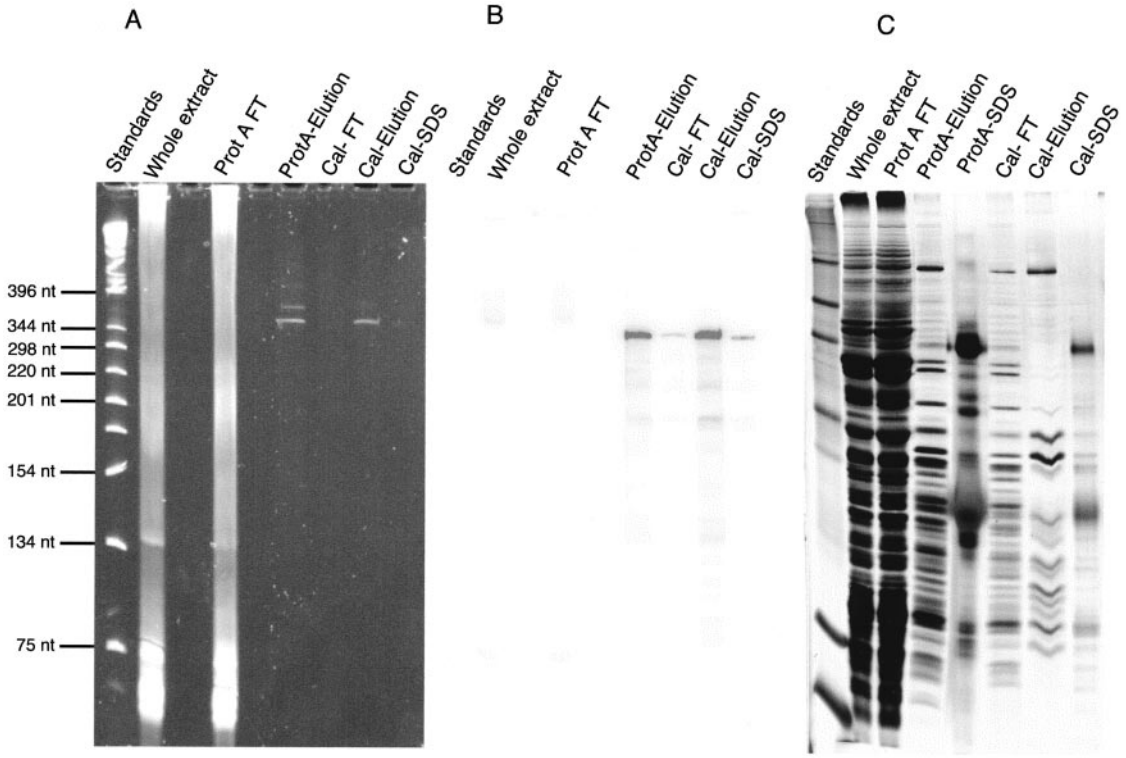

FIG. 2. **Tandem affinity purification profile of nuclear RNase MRP.** Aliquots of yeast extracts from the YSW1 strain were isolated from the final TAP fraction (*Cal-Elution*), the IgG affinity purification step (*Prot A-Elution*), unbound extracts (*Prot A FT* and *Cal-FT*), and tightly bound extracts (*Prot A-SDS* and *Cal-SDS*) for step-by-step analysis of the TAP purification process. *A*, analysis of RNA collected from extracts. RNA was extracted from aliquots using phenol/chloroform and precipitated. Total RNA from the indicated aliquots was separated on a 6% acyrlamide-7 M urea gel and examined by staining with ethidium bromide. *B*, Northern analysis of the RNA purified from the Pop4-TAP fusion strain. RNA from whole-cell extracts was run on a 6% acrylamide-7 M urea gel and transferred to a nylon membrane. The MRP RNA was visualized by hybridization with a ^{32}P-labeled probe containing the *NME1* gene. *C*, analysis of proteins purified. Proteins isolated from aliquots were separated on 12% SDS-PAGE gel and visualized using silver stain. Standards are the Invitrogen Mark12 protein standard. The sizes of these standards are provided in Fig. 4. *nt*, nucleotides

down product. Negative staining electron microscopy of the purified complex indicated homogeneous size particles of between 25 and 30 nm across.[2]

Identification of Purifying Proteins—Proteins isolated from many separate purifications were analyzed by SDS-PAGE. Consistently, there were at least 9–10 protein bands that separated between ~12 and 100 kDa, consistent with the size and number of proteins believed to be in the RNase MRP complex (Fig. 2). To identify which bands corresponded to known complex components, we took advantage of available antibodies. Western analyses were performed using antibodies for five of the established protein components of nuclear RNase MRP, namely, Snm1, Pop1, Pop3, Pop4, and Rpp1 (Fig. 3*B*). Proteins isolated from the final product of the TAP purification were run on SDS-PAGE and transferred to nitrocellulose. Each blot was incubated with an antibody corresponding to one of the individual protein components, probed with a secondary peroxidase-conjugated antibody, and visualized by chemiluminescence. In each case, the RNase MRP proteins were found in the final purified product, assisting in identifying the appropriate bands (Fig. 4). Antibodies against Pop1 identified a minor amount of breakdown product of this protein. These bands were absent in fresh preparations and increased in older preparations after several rounds of freezing and thawing. Antibodies to Snm1 also detected multiple bands near the same molecular mass. This is consistent with what is seen with these antibodies in whole-cell extracts (15). The multiple forms may be the result of proteolytic digestion or secondary modifications. We looked expressly for potential phosphorylation of this protein, but none was detected (data not shown).

Because antibodies are not available for all the known protein components of RNase MRP, we sought to identify the remaining components by mass spectrometry. Protein bands were excised from SDS-PAGE, digested with trypsin, and identified using MALDI-TOF mass spectroscopy (Table I). This identified with high confidence Pop1, Pop4 + tag, Rpp1, Snm1, Ylr145w, Pop3, Pop7, Pop5, Rpr2, Pop6, and Pop8 in the purified preparations of nuclear RNase MRP. All of these proteins were identified with a high degree of confidence in separate purifications. As a result, all the known protein components of RNase MRP, as well as Rpr2, a protein thought to be unique to the RNase P complex, were detected. Previous research suggested that RNase MRP contained only nine protein components. However, our analysis identified two additional proteins in the complex. Ylr145w is an essential gene that was recently identified in a genomic analysis as being part of the RNase P enzyme complex (30). Our data indicate that Rpr2p may also associate with RNase MRP. A summary of the MALDI-TOF mass spectroscopy data is shown in Table I.

Determination of the Stoichiometry of the RNase MRP Complex—In order to quantitate the relative amount of each of the proteins in the RNase MRP complex, we utilized the protein dye SPYRO Ruby. This fluorescent dye binds most proteins in a linear fashion independent of their amino acid composition. SDS-PAGE gels were stained with SPYRO Ruby and scanned using a Bio-Rad FluorS imager. Utilizing this approach we quantitated each of the identified bands relative to Pop1, which

[2] M. E. Schmitt and D. R. Mitchell, unpublished observations.

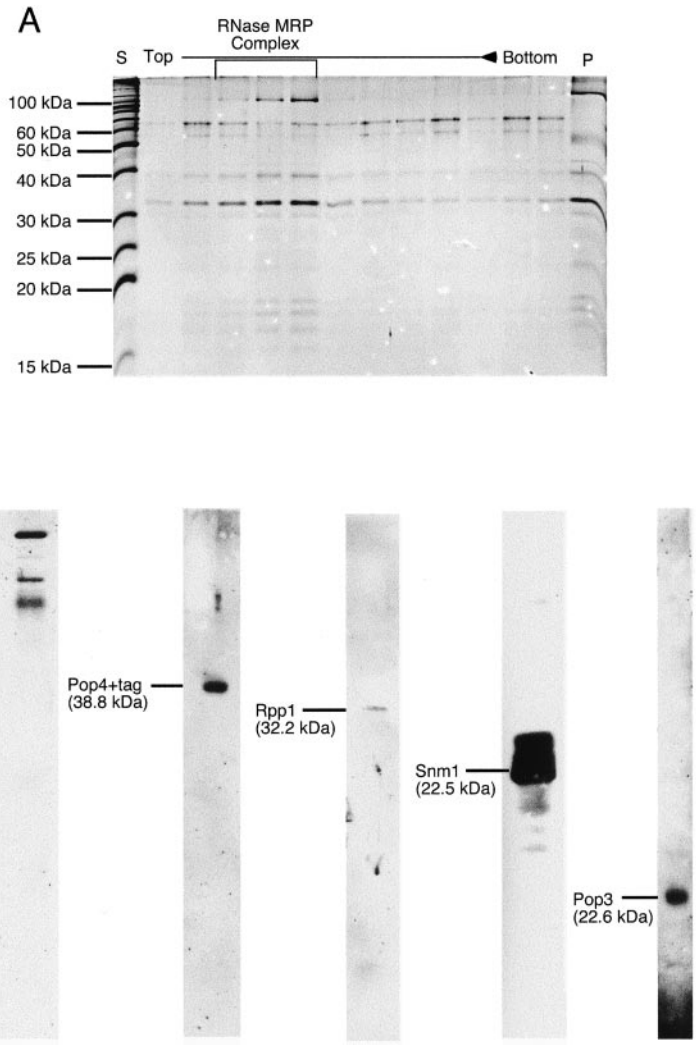

FIG. 3. **Identification of protein components in purified nuclear RNase MRP.** *A*, glycerol gradient centrifugation of the purified RNase MRP complex. The purified RNase MRP complex (*lane P*) was loaded onto a 15–30% glycerol gradient in Buffer Z+T and centrifuged as described previously (37). The sample was divided into 12 fractions, and aliquots were analyzed on a 15% SDS-PAGE gel and visualized with silver staining. *Lane S*, molecular weight standards, Invitrogen BenchMark Protein Ladder. *B*, two μg of purified RNase MRP proteins from the final TAP fraction were separated by SDS-PAGE through 15% polyacrylamide gels. Proteins were subsequently transferred to nitrocellulose membranes and probed with polyclonal antibodies against the indicated components (see "Materials and Methods"). The blots were probed with a secondary peroxidase-conjugated anti-rabbit antibody and visualized with chemiluminescence.

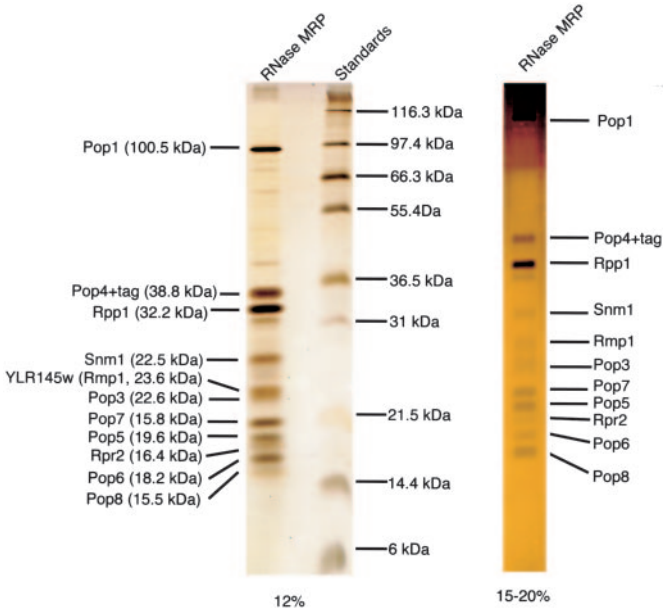

FIG. 4. **Purified RNase MRP proteins as identified by MALDI-TOF analysis.** Three μg of purified RNase MRP protein was separated by SDS-PAGE through either a 12% or 15–20% polyacrylamide gradient gel and visualized using silver staining (36). Individual bands were excised from gels, digested with trypsin, and identified using MALDI-TOF mass spectroscopy. The positions of RNase MRP protein components as identified by the MALDI-TOF data are indicated.

was given a value of 1. After imaging, the proteins were excised from gels, and their identity was confirmed using MALDI-TOF mass spectroscopy. These data are summarized in Table I.

Ylr145w Is a Component of RNase MRP, but Not RNase P—Our MALDI-TOF mass spectroscopy data indicate that the protein encoded by the uncharacterized open reading frame *YLR145w* may be a component of nuclear RNase MRP. In an attempt to confirm that this gene product associates with RNase MRP and P, we tried to precipitate the corresponding RNAs with a tagged version of the Ylr145w protein. The *POP4*::*TAP*-tagged strain was used as a positive control. Whole-cell lysates were incubated with IgG beads. RNAs extracted from the beads were separated on 6% acrylamide-7 M urea gels, transferred to nylon membranes, and probed for RNase P RNA or RNase MRP RNA (Fig. 5). The Pop4::TAP tag co-immunoprecipitates the RNase MRP RNA and the RNase P RNA. However, a Ylr145w::TAP tag specifically co-immunoprecipitates the RNase MRP RNA but not the RNase P RNA. This suggests that the protein encoded by *YLR145w* is only associated with the RNase MRP complex, and not the RNase P complex. For this reason, the gene was named *RMP1*, for RNase MRP protein 1.

Homologues of the YLR145w Gene—Nearly all of the RNase MRP components are highly conserved throughout eukaryotes. We examined whether this was also true of the Ylr145w protein. We did a BLAST search in an attempt to identify homologues of the Ylr145w protein. As shown in Fig. 6, we discovered that proteins in a wide variety of yeast and fungi species

TABLE I
Summary of nuclear RNase MRP proteins identified by MALDI-TOF analysis

The number of peptides identified in the analysis is given. Stoichiometry was determined from scans of gels stained with SPYRO-Ruby and is relative to the Pop1 protein (see "Materials and Methods"), which is given a value of 1.

Component	Mass/subunit	No. of peptides >500 Da identified	Theoretical no. of peptides between 500 Da and 4 kDa	Total mass coverage	Stoichiometry
	kDa			*%*	*Rounded (actual)*
Pop1	100.4	56	71	57	1
Pop4+tag	38.8	23	35	52	3 (2.95)
Rpp1	32.2	11	22	28	3 (3.33)
Rmp1	23.6	5	14	18	2 (1.73)
Snm1	22.5	11	15	27	2 (2.38)
Pop3	22.6	3	14	19	2 (1.90)
Pop5	19.6	7	15	28	4 (4.26)
Pop6	18.2	4	11	19	4 (3.92)
Rpr2	16.4	4	12	31	2 (2.20)
Pop7	15.8	2	12	14	4 (3.84)
Pop8	15.5	4	10	32	4 (4.21)
NME1 RNA	109.7				1?

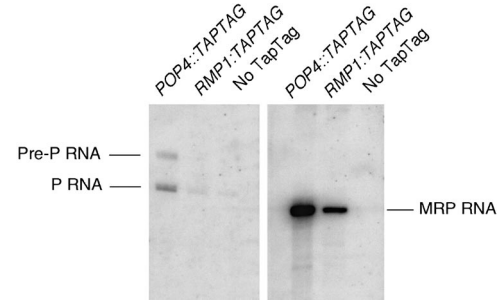

FIG. 5. **Immunoprecipitation of RNase MRP RNA by TAP-tagged Rmp1.** Whole-cell extracts from a wild-type strain (*No TapTag*) and a *RMP1:TAP*-tagged strain were incubated with IgG beads for 60 min and then washed several times. RNA was extracted from the beads. The TAP-tagged Pop4 strain was used as a positive control. Equal amounts of RNA were separated on 6% acrylamide-7 M urea gels and analyzed by Northern analysis. *Left panel*, RNase P RNA visualized by hybridization with a ^{32}P-labeled probe corresponding to a 450-bp fragment containing the *RPR1* gene. *Right panel*, RNase MRP RNA visualized by hybridization with a ^{32}P-labeled probe corresponding to a 471-bp fragment containing the *NME1* gene (15).

share similarity within three regions of the Ylr145w protein. Regions of similarity were concentrated in the amino terminus, a central domain, and the carboxyl terminus. The discovery of putative homologues to Ylr145w in a wide variety of fungi indicates that this is a conserved component of the RNase MRP complex. Utilizing a conserved consensus, we were able to detect a homologue in the *Arabidopsis* genome (locus B96546). Homologues were not detected in any metazoans. The homologous sequences do not correspond to any known protein domains.

Isolation of a Temperature-conditional Mutant of rmp1—In an effort to further characterize Rmp1p, we created a temperature-sensitive rmp1 mutant. Random PCR mutagenesis of the *RMP1* gene was performed. Potential mutants were screened for cold and temperature sensitivity at 25 °C and 37 °C. One strain in particular exhibited a strong temperature-sensitive growth phenotype (Fig. 7). The rmp1-6 strain grows well at 25 °C but grows only very slowly at 37 °C.

We confirmed that the growth phenotype of the rmp1-6 strain we observed was due to the presence of the mutagenized *rmp1* gene. A single point mutation was identified in the *rmp1* gene. This mutation changes cysteine 103 to an arginine (Fig. 6). The mutation sits within the conserved central domain of Rmp1, which may explain why this mutant exhibits a temperature-sensitive growth phenotype.

Processing of 5.8S rRNA in the rmp1-6 Mutant—The temperature-sensitive growth phenotype of the rmp1-6 mutant suggests that the mutation affects the function of the RNase MRP complex. Mutations in components of the complex, including *nme1*, *pop1*, and *snm1*, have been shown to result in a defect in the processing of 5.8S rRNA (2, 11, 15). Two species of 5.8S rRNA exist in yeast; they differ in length by only 7 nucleotides and are generated through independent processing pathways. The smaller species, which is generated in a MRP-dependent manner, is ~8–10-fold more abundant than the larger species. Loss of function of components of the RNase MRP complex results in decreased processing of the smaller 5.8S rRNA and an increase in the larger RNase MRP-independent species. The rmp1-6 strain was grown at 30 °C until it reached exponential phase and then shifted to non-permissive temperature for 4 h. Total RNA was isolated from cells before and after the shift. As shown in Fig. 8, a defect in 5.8S rRNA processing was observed in the rmp1-6 mutant at both the permissive and non-permissive temperatures. This change in the ratio of 5.8S rRNA species correlates well with the phenotype seen in other RNase MRP mutants (2, 11, 20, 31). These results demonstrate that Rmp1p is required for the function of the RNase MRP enzyme in rRNA processing.

The Stability of RNase MRP RNA in the rmp1-6 Mutant—The RNA product of the *NME1* gene is essential and is required for the function of the RNase MRP complex. If Rmp1 is associated with the RNase MRP complex, then mutations in Rmp1p could compromise the stability of the RNase MRP RNA, leading to a defect in rRNA processing. To test this possibility, Northern analyses were performed on RNA isolated from the rmp1-6 mutant at permissive and non-permissive temperatures. As shown in Fig. 8, MRP RNA was stable in the rmp1-6 strain under all conditions. This result is similar to what is seen in mutations in the RNase MRP-specific protein Snm1 (20). It also indicates that Rmp1 is probably not a core protein subunit of the complex such as Pop1 that loses stability of the RNA rapidly upon depletion or mutation (11). In addition, the RNase P RNA was found to be stable in the rmp1-6 mutant strain (Fig. 8).

The Processing of tRNA in the rmp1-6 Mutant—The RNase P complex is involved in the processing of the 5′ ends of tRNAs. Analysis of total steady-state levels of tRNAs indicated that there were no gross changes in the levels of tRNAs, as would be expected in an RNase P mutant (Fig. 8) (11). We also performed Northern analyses on RNA isolated from the rmp1-6 mutant to identify low abundant tRNA precursors. Blots were probed for the presence of a pre-tRNA$_{Arg}$. As shown in Fig. 8, only mature pre-tRNA$_{Arg}$ is seen in the rmp1-6 mutant strain at both the permissive and non-permissive temperatures. There is no accumulation of tRNA precursors, as would be expected in an

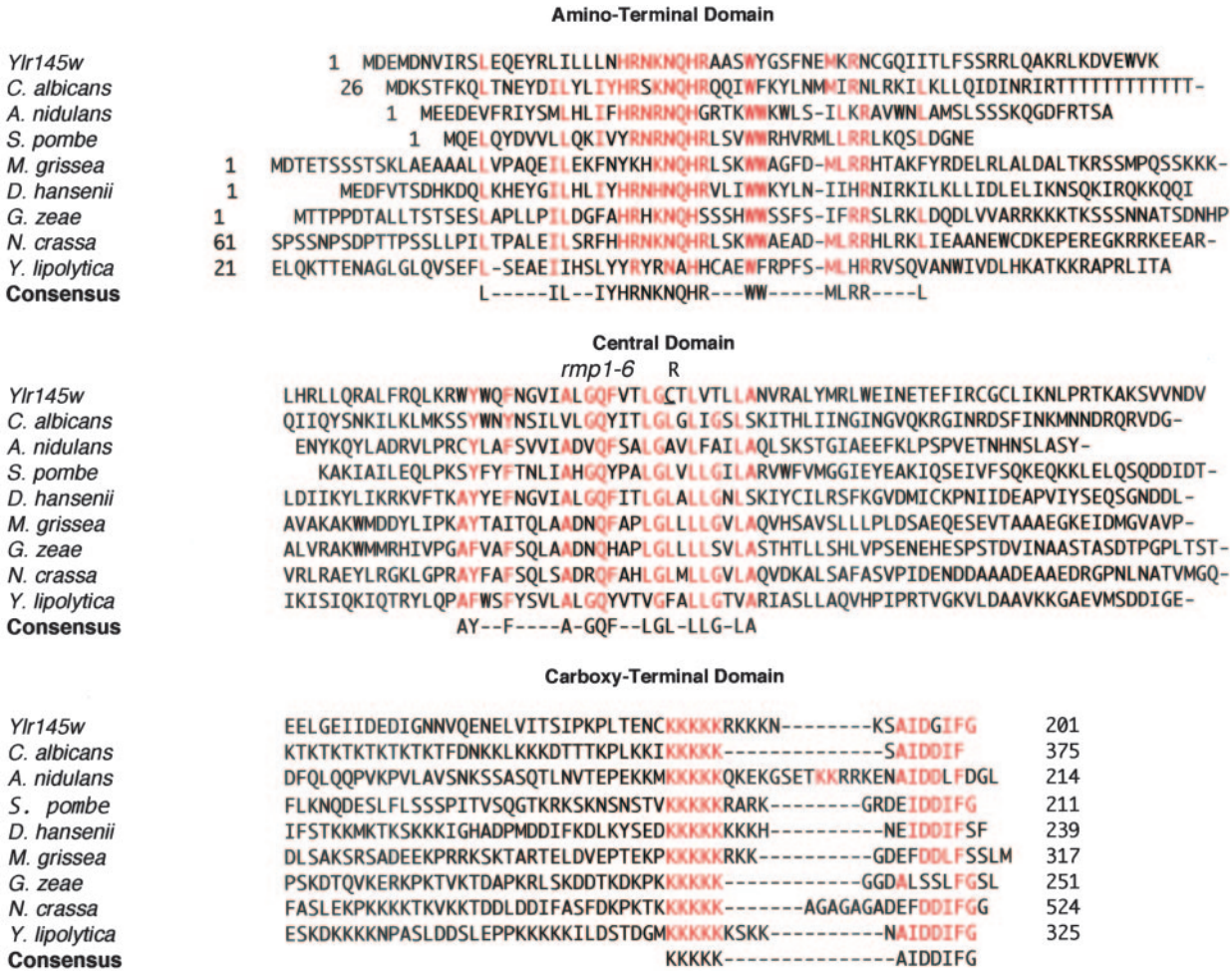

FIG. 6. **Alignment of YlR145w with identified homologues.** A NCBI BLAST search was conducted in an attempt to identify potential YlR145w homologues. Three putative domains of homology in the amino terminus, the central region, and the carboxyl terminus were identified in all of the proteins found. The position of the single amino acid change in the *rmp1-6* mutation is indicated. Sequences are from NCBI (accession no.): *Debaryomyces hansenii* (XP 460019); *Candida albicans* (EAL03521); *Aspergillus nidulans* (AN6495); *Schizosaccharomyces pombe* (NP 594378); *Yarrowia lipolytica* (XP 503555); *Magnaporthe grisea* (EAA46485); *Gibberella zeae* (EAA67234); and *Neurospora crassa* (XP 326059).

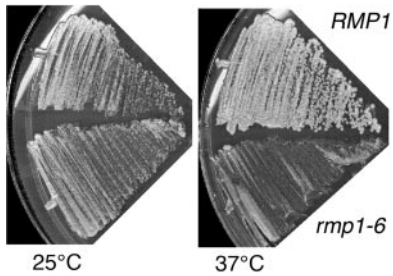

FIG. 7. **Growth phenotype of the rmp1-6 mutant.** Yeast strain carrying a wild-type *RMP1* on a *URA3*/*CEN* plasmid and a yeast strain carrying the *rmp1-6* mutation on a *LEU2*/*CEN* plasmid were plated at the indicated temperature onto YPD plates and photographed after 1 day.

RNase P mutant. These results confirm that Rmp1 is not a component of the RNase P complex.

DISCUSSION

We were able to purify RNase MRP to apparent homogeneity using tandem affinity purification of Pop4 and its associated proteins. The *POP4* locus is an ideal candidate for integration of the TAP tag because Pop4 is an integral component of the RNase MRP complex. Depletion of Pop4p results in the loss of RNase MRP RNA. Not only does Pop4p interact with most of the shared protein components of RNase MRP and RNase P, but it has also been shown to interact with the only unique protein component of RNase MRP identified so far, Snm1p (15). In addition, Pop4 is a relatively stable protein. Although Pop1p is another integral protein component of the RNase MRP complex that could be considered a potential target for the TAP cassette, Pop1p is relatively unstable, and Western analysis using an anti-Pop1 antibody confirms that multiple breakdown products are present (14, 32, our study). In addition, carboxyl-terminal fusions to the Pop1p have been found to be lethal (11).

Judging by RNA analysis of the purified RNase MRP, the intact complex was contaminated with <1% of RNase P. The use of mild salt conditions to obtain whole-cell yeast extracts creates an environment more favorable for the isolation of RNase MRP than RNase P. Previous work aimed at isolating RNase P has shown that high salt conditions are amenable for purification and extraction of this complex (14). Although it appears that some RNase P RNA co-purifies through the first column, the use of a second affinity purification matrix, calmodulin, eliminates detectable RNase P RNA. Purified RNase MRP is probably altered very little from its natural state. Pop4 isolated from the final TAP fraction only retains the calmodulin-binding portion of the TAP cassette. Previous studies using the TAP fusion cassette suggest that the calmodulin-binding domain does not affect the structure or function of most purified products (19). Indeed, our purified RNase MRP is fully active in cleaving *in vitro* the A3 site in a yeast rRNA and the

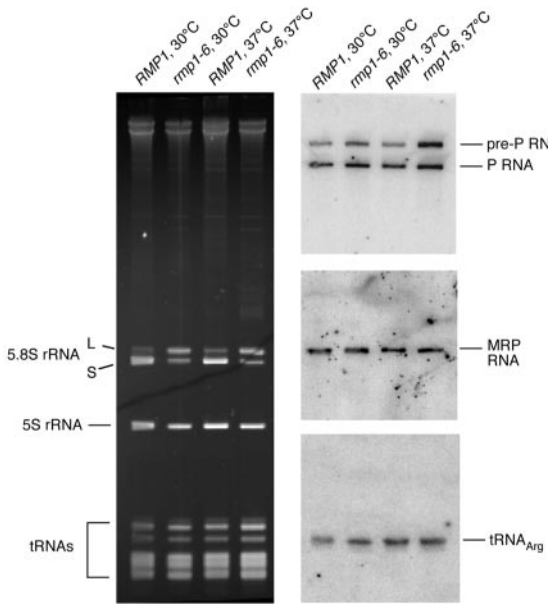

FIG. 8. **RNA analysis of the rmp1-6 mutant.** Yeast strains were grown to A_{600} = 0.4 at 30 °C in YPD and then shifted to 37 °C for 4 h or maintained at 30 °C as indicated. Cells were collected, and total RNA was isolated before and after the shift. *Left panel*, equal amounts of RNA were separated on a 6% acrylamide-7 M urea gel and stained with ethidium bromide. The locations of 5.8S(S), 5.8S(L), and 5S rRNA, as well as tRNA species, are shown. *Right panels*, RNA was separated on 6% acrylamide-7 M urea gels and transferred to nylon membranes. *Top right panel*, pre-RNase P and RNase P RNA were visualized by hybridization with a ^{32}P-labeled 460-bp probe containing the *RPR1* gene. *Middle right panel*, RNase MRP RNA was visualized using a ^{32}P-labeled probe corresponding to the coding region for *NME1*. *Bottom right panel*, tRNA$_{Arg}$ was visualized by hybridization with a ^{32}P-labeled 189-bp probe that encompasses the *tR(ACG)L* precursor.

5′-UTR of *CLB2* substrate (10). This evidence suggests that the purified RNase MRP is an active, intact complex.

Based on our analysis of purified RNase MRP, most protein components associate at more than one copy per complex, whereas the MRP RNA and Pop1p may be present in only a single copy. Previous examination of the stoichiometry of the RNase P complex suggests that RNase P RNA is also present in a single copy per complex (33). However, no other stoichiometric analysis of RNase P or MRP has been performed. Reason would dictate that the RNase P complex that shares 10 proteins with the RNase MRP complex would contain similar stoichiometry of the shared subunits. Differences may be in the numbers of unique protein subunits, Rmp1, Snm1, and Rpr2. Many of the proteins have been shown to interact with themselves in both yeast and humans, consistent with them being present at more than one copy per complex (32, 34, 35). We have been able to estimate the relative stoichiometry of each of the protein subunits to each other. However, these estimations do not preclude the complex from being a dimer, trimer, or more, with multiple copies of the MRP RNA present.

Rpr2p was identified by MALDI-TOF mass spectrometry to be in our purified RNase MRP complex. However, Rpr2p is considered to be a unique protein component of RNase P, based on immunoprecipitation and depletion experiments. Rpr2 has been shown to precipitate only RNase P RNA and not RNase MRP RNA (14). In addition, depletion of Rpr2p results in preferential loss of RNase P RNA (14). Based on our data, Rpr2p is present in purified preparations of RNase MRP at greater levels than can be explained by contamination alone. In part, Rpr2p may be co-purified because it has been shown to interact strongly with Pop4p (32).

We propose that Rpr2p serves an analogous and overlapping function with Snm1p in the RNase MRP complex. Both Rpr2p and Snm1p have been shown to interact strongly with themselves and with Pop4p, suggesting that they might associate as dimers (32). The two proteins share sequence similarity and are probably evolutionarily derived from the same gene. In addition, depletion of Rpr2p does result in a 2-fold reduction in RNase MRP RNA levels (14). The presence of Rpr2p in our purified RNase MRP could be explained by the existence of Snm1p-Rpr2p dimers. Based on the subunit quantitation, Snm1 is present in equal concentrations to Rpr2, and the two could form Snm1p-Rpr2p dimers that do not compromise the function of the RNase MRP complex. In the absence of Rpr2, the Snm1 protein can functionally replace the Rpr2 protein. Indeed, Rpr2 may be able to partially replace Snm1 because mutations in the *SNM1* gene that produce very little protein are still viable (20).

We also provide evidence that a protein encoded by the uncharacterized open reading frame *YLR145w* is present in purified RNase MRP. MALDI-TOF mass spectrometry analysis of purified protein components revealed the new potential protein component. The fact that a TAP-tagged version of YlR145w precipitates RNase MRP RNA, but not RNase P RNA, suggests that this protein is unique to RNase MRP. In support, the *rmp1-6* mutation confers a 5.8S rRNA processing defect, but not a tRNA processing defect. Previous research aimed at identifying the function of uncharacterized open reading frames showed that TAP-tagged YlR145w precipitated most of the shared protein components of the RNase MRP and RNase P, but no further analysis was performed (30). Based on our evidence, we believe that *YLR145w* encodes a new unique protein component of RNase MRP, Rmp1p.

We have uncovered putative homologues of Ylr145w. Nearly all of the MRP components including the RNA have conserved homologues in higher eukaryotes (38). More research will be required to further characterize the association of Rmp1p with the RNase MRP complex. Rmp1p is required for proper rRNA processing, but we have yet to determine whether Rmp1 is also required for other functions of RNase MRP. Indeed, the requirement of RNase MRP to perform multiple processing events may require specialized proteins for substrate recognition or regulation. RNase P may also contain other yet to be identified proteins that are required for its specific cellular processes.

The availability of large amounts of highly purified RNase MRP will open the door to structural analysis of this complex. The purification level is high enough to allow for both cryo-electron microscopy and crystallization of the complex for x-ray diffraction studies. In addition, it will allow for easy analysis of other potential substrates of the complex and for identification of post-translational modifications that may regulate the activity of RNase MRP.

Acknowledgments—We thank Jason Aulds, Tina Gill, Qiaosheng Lu, and David Amberg for comments and helpful discussions during preparation of the manuscript. We also thank Lorraine Symington and Bertrand Séraphin for stains and plasmids.

REFERENCES

1. Reimer, G., Raska, I., Scheer, U., and Tan, E. M. (1998) *Exp. Cell Res.* **176,** 117–128
2. Schmitt, M. E., and Clayton, D. A. (1993) *Mol. Cell. Biol.* **13,** 7935–7941
3. Henry, Y., Wood, H., Morrissey, J. P., Petfalski, E., Kearsey, S., and Tollervey, D. (1994) *EMBO J.* **13,** 2452–2463
4. Chu, S., Archer, R. H., Zengel, J. M., and Lindahl, L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **18,** 659–663
5. Lygerou, Z., Allmang, C., Tollervey, D., and Séraphin, B. (1996) *Science* **272,** 268–270
6. Chang, D. D., and Clayton, D. A. (1987) *Science* **235,** 1178–1184
7. Topper, J. N., Bennett, J. L., and Clayton, D. A. (1992) *Cell* **70,** 16–20
8. Chang, D. D., and Clayton, D. A. (1987) *EMBO J.* **6,** 409–417
9. Lee, D. Y., and Clayton, D. A. (1998) *J. Biol. Chem.* **273,** 30614–30621
10. Gill, T., Cai, T., Aulds, J., Wierzbicki, S., and Schmitt, M. E. (2004) *Mol. Cell. Biol.* **24,** 945–953

11. Lygerou, Z., Mitchell, P., Petfalski, E., Séraphin, B., and Tollervey, D. (1994) *Genes Dev.* **8,** 1423–1433
12. Dichtl, B., and Tollervey, D. (1997) *EMBO J.* **16,** 417–429
13. Stolc, V., and Altman, S. (1997) *Genes Dev.* **11,** 2414–2425
14. Chamberlain, J. R., Lee, Y., Lane, W. S., and Engelke, D. R. (1998) *Genes Dev.* **12,** 1678–1690
15. Schmitt, M. E., and Clayton, D. A. (1994) *Genes Dev.* **8,** 2617–2628
16. Forster, A. C., and Altman, S. (1990) *Science* **249,** 783–786
17. Schmitt, M. E., Bennett, J. L., Dairaghi, D. J., and Clayton, D. A. (1993) *FASEB J.* **7,** 208–213
18. Chamberlain, J. R., Pagan-Ramos, E., Kindelberger, D. W., and Engelke, D. R. (1996) *Nucleic Acids Res.* **24,** 3158–3166
19. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Séraphin, B. (1999) *Nat. Biotechnol.* **17,** 1030–1032
20. Cai, T., Reilly, T. R., Cerio, M., and Schmitt, M. E. (1999) *Mol. Cell. Biol.* **19,** 7857–7869
21. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY
22. Cai, T. (2000) *Cell Cycle Regulation by an Endoribonuclease.* Ph.D. thesis, Upstate Medical University, Syracuse NY
23. Muhlrad, D., Hunter, R., and Parker, R. (1992) *Yeast* **2,** 79–82
24. Gietz, R. D., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 527–534
25. Sikorski, R. S., and Boeke, J. D. (1991) *Methods Enzymol.* **194,** 302–318
26. Cai, T., Aulds, J., Gill, T., Cerio, M., and Schmitt, M. E. (2002) *Genetics* **161,** 1029–1042
27. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) Nucleic Acids Res. **18,** 3091–3092
28. Shadel, G. S., Buckenmeyer, G. A., Clayton, D. A., and Schmitt, M. E. (2000) *Gene* (*Amst.*) **245,** 175–184
29. Huang, K. N., and Symington, L. S. (1993) *Mol. Cell. Biol.* **6,** 3125–3134
30. Hazbun, T. R., Malmstrom, L., Anderson, S., Graczyk, B., Fox, B., Riffle, M., Sundin, B. A., Aranda, J. D., McDonald, W. H., Chiu, C., Syndsman, B. E., Bradley, P., Muller, E., Fields, S., Baker, D., Yates, J. R., and Davis, T. N. (2003) *Mol. Cell. Biol.* **12,** 1353–1365
31. Li, X., Zaman, S., Langdon, Y., Zengel, J. M., and Lindahl, L. (2004) *Nucleic Acids Res.* **32,** 3703–3711
32. Houser-Scott, F., Xiao, S. H., Millikin, C. E., Zengel, J. M., Lindahl, L., and Engelke, D. R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2684–2689
33. Srisawat, C., and Engelke, D. R. (2001) *RNA* (*N. Y.*) **7,** 632–641
34. Welting, T. J. M., van Venrooij, W. J., and Pruijn, G. J. M. (2004) *Nucleic Acids Res.* **32,** 2138–2146
35. Li, Y., and Altman, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 441–444
36. Wray, W., Boulikas, T., Wiay, V. P., and Huncock, R. (1981) *Anal. Biochem.* **118,** 197–203
37. Cai, T, and Schmitt, M. E. (2001) *Methods Enzymol.* **342,** 135–142
38. Aulds, J., Cai, T., and Schmitt, M. E. (2002) *Recent Res. Dev. Mol. Cell. Biol.* **3,** 371–378