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RPM2, Independently of Its Mitochondrial RNase P Function, Suppresses an *ISP42* Mutant Defective in Mitochondrial Import and Is Essential for Normal Growth

C. KENNETH KASSENBROCK,[1]† GUO-JIAN GAO,[2] KATHLEEN R. GROOM,[2] PAVOL SULO,[2]‡ MICHAEL G. DOUGLAS,[1]§ AND NANCY C. MARTIN[2]*

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina,[1] *and Department of Biochemistry, School of Medicine, University of Louisville, Louisville, Kentucky*[2]

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***RPM2* is identified here as a high-copy suppressor of *isp42-3*, a temperature-sensitive mutant allele of the mitochondrial protein import channel component, Isp42p. *RPM2* already has an established role as a protein component of yeast mitochondrial RNase P, a ribonucleoprotein enzyme required for the 5′ processing of mitochondrial precursor tRNAs. A relationship between mitochondrial tRNA processing and protein import is not readily apparent, and, indeed, the two functions can be separated. Truncation mutants lacking detectable RNase P activity still suppress the *isp42-3* growth defect. Moreover, *RPM2* is required for normal fermentative yeast growth, even though mitochondrial RNase P activity is not. The portion of *RPM2* required for normal growth and suppression of *isp42-3* is the same. We conclude that *RPM2* is a multifunctional gene. We find Rpm2p to be a soluble protein of the mitochondrial matrix and discuss models to explain its suppression of *isp42-3*.**

Cellular membranes impose a permeability barrier for hydrophilic molecules that is overcome by specialized transport systems. In eukaryotic cells, membrane-bound organelles require transport systems both for small solutes and for the protein constituents of the organelle.

Although mitochondria contain their own genome and protein-synthetic machinery, relatively few proteins are encoded by the organelle DNA. Most mitochondrial proteins are made in the cytosol as precursors which are imported and processed by a specialized import apparatus. The machinery used by mitochondria to import proteins from the cytosol is not completely defined; nevertheless, significant progress has been made recently in identifying components of the protein translocation apparatus (9, 23, 27).

In an effort to identify additional components of the protein transport machinery of mitochondria, a genetic search in the yeast *Saccharomyces cerevisiae* was initiated (13). The starting point for this search was the gene *ISP42*, which encodes a 42-kDa protein which has been shown to form part of the protein translocation pore in the mitochondrial outer membrane (2). A *Neurospora crassa* homolog, *MOM38*, was independently identified as an import channel component in that species (14). We generated temperature-sensitive mutants of *ISP42* and used these mutants to search for normal yeast genes which could suppress the temperature-sensitive phenotype if present in the cell at an increased copy number. Such genes are termed high-copy suppressors, and may encode products that physically interact with Isp42p. A novel gene, *ISP6*, was identified in this manner and shown to encode a new component of the outer mitochondrial membrane protein translocation apparatus (13).

We now describe the identification and cloning of another high-copy suppressor of a temperature-sensitive *ISP42* mutant and report the surprising finding that it is allelic to a previously described gene, *RPM2. RPM2* encodes a protein component of the mitochondrial RNase P enzyme (20). RNase P is required for maturation of tRNAs, cleaving 5′ leader sequences from tRNA precursors to yield the mature tRNA and the 5′ leader. A number of procaryotic RNase P enzymes have been studied in molecular detail and shown to be ribonucleoproteins, containing a single catalytic RNA molecule and a protein subunit of about 14 kDa (1, 6). Rpm2p has no obvious homology with the procaryotic protein subunits, and it is almost 10 times larger (5). The only other eukaryotic protein associated with RNase P activity with a known primary sequence is the product of the *POP1* gene (18). Its role in the nuclear RNase P of *S. cerevisiae* is not yet known.

A connection between mitochondrial protein import and maturation of mitochondrial tRNAs is not readily apparent, so it is surprising and puzzling that *RPM2* should be recovered as an efficient suppressor of *ISP42* mutants. We demonstrate that the suppression is independent of mitochondrial RNase P activity. Deletion analysis of *RPM2* reveals that only the amino-terminal half of the open reading frame is required for this suppression. Finally, our experiments show that deletion of the entire gene causes a severe growth defect. Thus, *RPM2* is a multifunctional gene, required for mitochondrial tRNA processing and for another, as yet undefined function, essential for normal growth.

MATERIALS AND METHODS

The details of constructing the temperature-sensitive alleles of *ISP42*, yeast growth and transformation, and screening the yeast genomic library for high-copy suppressors have been previously described (13). The yeast strain KKY3.3 (previously called KKY3-ts#3) is derived from KKY3 by replacement of the plasmid carrying wild-type *ISP42*, pRS316-ISP42, with the plasmid bearing the temperature-sensitive *isp42-3* allele, pRS314-isp42-3, as described previously

* Corresponding author. Mailing address: Department of Biochemistry, School of Medicine, University of Louisville, Louisville, KY 40292. Phone: (502) 852-7797. Fax: (502) 852-6222.

† Present address: Department of Biology, Colorado State University, Fort Collins, CO 80523.

‡ Present address: Institute of Microbiology and Genetics, University of Vienna, A-1030 Vienna, Austria.

§ Present address: Sigma Diagnostics, St. Louis, MO 63103.

(13). Northern (RNA) analysis was performed as described elsewhere (20) by using a probe made from pMM6 transcribed with T3 polymerase under conditions recommended by the supplier (Gibco-BRL).

Subcloning of 3NS57. The original YEp24-based plasmid recovered as a high-copy suppressor of *isp42-3* was termed 3NS57. 3NS57 was cut with *Xho*I and *Kpn*I, and the 5.67-kb fragment containing the *RPM2* gene was subcloned into the vector p306-2μ (13) cut with the same enzymes to generate p57XK. Plasmid p57HX was generated as follows. 3NS57 was cut with *Xho*I and *Hin*dIII, and the 3.67-kb fragment was subcloned into pBluescript IIKS^{-} (Stratagene) to generate pBS-57HX. The insert was then excised from this vector by using *Xho*I and *Eag*I, and subcloned into p306-2μ cut with the same enzymes. Plasmids p57H7, p57H10, and p57H16 were derived from p57HX as follows. p57HX was cut with *Sma*I and *Sac*I and digested with exonuclease III by standard methods (26). Samples taken at various time points during the reaction were treated with mung bean exonuclease and then ligated to generate a series of nested deletions extending in from the *Hin*dIII side of the insert. Plasmid p57XX consists of a 2.24-kb *Xho*I-*Xba*I fragment from 3NS57 cloned into p306-2μ and was made by cutting pBS-57HX with *Xba*I, religating, and then cutting the insert out of the resulting plasmid with *Xho*I and *Eag*I and subcloning into p306-2μ cut with the same enzymes.

Gene disruptions. The disruption of *RPM2* at the *Hpa*I site has been described previously (20). An insertional disruption of *RPM2* at the *Sph*I site was made as follows. pBS-57HX was digested with *Sph*I, and the resulting ends were filled in by the Klenow enzyme. A 1.3-kb *Bam*HI-*Xho*I fragment containing the *HIS3* gene was made blunt by the Klenow enzyme and ligated to the above to generate p57HX::HIS3. p57HX::HIS3 was cut with *Xho*I and *Eag*I to release the insert and then used to transform diploid W303 cells to His^{+} prototrophy. The resultant diploid strain, heterozygous for *rpm2*::*HIS3*, was sporulated, and tetrads were dissected as shown in Fig. 5.

An extensive deletion of *RPM2* was made in plasmid YEp352/RPM2, which was constructed by ligating three DNAs. Fragment 1 was derived by cloning a 3.4-kb *Eco*RI fragment containing the upstream and amino-terminal coding sequences of *RPM2* into pBluescriptII KS^{+} (Stratagene) and then cutting this plasmid in the polylinker region with *Hin*dIII and in *RPM2* at the *Sph*I site (position 1651). Fragment 2 was an *Sph*I-*Bam*HI fragment spanning nucleotides 1651 to 4900 of *RPM2*. YEp352 was cut with *Hin*dIII and *Bam*HI, and the three fragments were ligated together. Removal of the sequence coding for amino acids 144 to 1172 of *RPM2* was accomplished by cutting this plasmid with *Bgl*II and *Xba*I; ends were filled in by the Klenow enzyme and a 2.2-kb *Hpa*I fragment containing the *LEU2* gene from YEp13 was inserted. The resultant plasmid, YEp352/BXLEU2, was digested with *Bam*HI, and a 3.6-kb fragment containing the gene deletion was introduced into the diploid yeast W303 by electroporation. Positive Leu^{+} transformants with integrations at the *RPM2* locus confirmed by Southern blot analysis were transformed with YEp352/RPM2, thus providing wild-type *RPM2* on an exogenously replicating plasmid. Leu^{+} Ura^{+} transformants were sporulated, tetrads were dissected, and Leu^{+} Ura^{+} spores were selected to generate haploid BXLEU2/RPM2 cells. The phenotype of this deletion was identical to that of a complete coding-sequence deletion.

A complete and precise coding sequence deletion of *RPM2* was made as follows. PCR was performed with p57-XK as template and primers M13 forward (GTAAAACGACGGCCAGT) and 3′-B-57-588 (CGCGGATCCTTTCGTTT GTGTATGCTTGTT). The product of this reaction was cut with *Xho*I and *Bam*HI and subcloned into pSP72 (Promega). A second PCR was performed with p57-XK as template and primers 5′-B-57-4198 (GCCGGATCCTTTACA ATTTAATATTTTATTT) and 57-3′-Kpn (CGAGGTACCGCCGTCAAGGCG TAATCA). The product of this reaction was cut with *Bam*HI and *Kpn*I and cloned into the above plasmid cut with the same enzymes to generate pSP72-Δ57. pSP72-Δ57 contains an insert beginning at an *Xho*I site 588 bp upstream of the coding region of *RPM2* and extending 4,640 bp downstream to a *Bgl*I site but missing the entire coding sequence beginning with the initiation methionine and including the stop codon. A new *Bam*HI site replaces the coding sequence. A 1.3-kb *Bam*HI-*Xho*I fragment containing the yeast *HIS3* gene was filled in at both ends with the Klenow enzyme and ligated to pSP72-Δ57, which had been digested with *Bam*HI and made blunt ended by the Klenow enzyme, thus generating plasmid pSP72-Δ57::HIS3. pSP72-Δ57::HIS3 was next cut with *Eco*RI, which cuts once in the insert 24 bp downstream of *Xho*I and once in the polylinker region on the other side of the insert, and used to transform the diploid W303 yeast. His^{+} transformants were screened for correct integration into the genomic *RPM2* site by PCR and confirmed by Southern blotting to generate the diploid strain, KKY50, which is heterozygous Δ*rpm2*::*HIS3*/+.

Plasmid shuffle. A 4,639-bp *Msc*I fragment of Yep352/RPM2 encoding the entire *RPM2* gene was cloned into *Pvu*II-digested pRS313 (28), a centromeric vector bearing the nutritional marker *HIS3*. This plasmid, named pRS313/RPM2, was introduced into haploid BXLeu2/RPM2 cells by electroporation. Individual transformants selected on SC-leu-his plates were suspended in water and plated in 10-fold serial dilutions on SC-leu-his plates with or without 1 mg of 5-fluoro-orotic acid (5-FOA) per ml.

Generation of antibodies. Antibodies to Rpm2p were raised against fusion proteins produced in *Escherichia coli*. An antibody to the amino-terminal region of Rpm2p has been previously described (5). An antibody to the carboxyl-terminal 273 amino acids of Rpm2p was made as follows. PCR was performed with p57XK as template and primers 57-929-Bam (CGCGGATCCAAATCTT GTATCAGGTCT) and 57-C-Sal (AGATCTCGAGTCGACTCATGCTTGAA GAGGCTT). The resultant product was cut with *Bam*HI and *Sal*I and cloned into pATH3 (16), cut with the same enzymes. This plasmid was transformed into the *E. coli* RR1 and induced as described elsewhere (16). The fusion protein was purified from the insoluble fraction by preparative sodium dodecyl sulfate (SDS) gel electrophoresis, excised from the gel, electroeluted, and used to immunize rabbits.

Epitope-tagged Rpm2p. Rpm2p containing the c-*myc* epitope EQKLISEEDL recognized by the monoclonal antibody 9E10 (7) at the carboxyl terminus was generated as follows. PCR was performed with p57XK as template and primers 57-20 (TTGAACCCTTTCACTCCT) and 57-CEPI-3′ (ATTAAATTGTAAAG GTCACAAGTCTTCTTCAGAAATAAGCTTTTGTTCTGCTTGAAGAG GCTTGCT). The 284-bp product was gel purified and designated product 1. A second PCR was performed with p57XK as template and primers 57-CEPI-5′ (AGCAAGCCTCTTCAAGCAGAACAAAAGCTTATTTCTGAAGA AGACTTGTGACCTTTACAATTTAAT) and 57-3′-Kpn (described above). The 502-bp product was gel purified and designated product 2. A third PCR was performed with a mixture of product 1 and product 2 as template and primers 57-20 and 57-3′-Kpn. The 721-bp product was cut with *Xba*I and *Kpn*I and ligated to the 10.7-kb band resulting from cutting p57XK with *Kpn*I and partially digesting with *Xba*I. The resultant plasmid is termed p306-2μ-57epi. The DNA sequence from the *Xba*I site through the end of the coding sequence was confirmed to be correct by DNA sequencing. This plasmid was cut with *Xho*I and *Kpn*I, and the insert was subcloned into pRS314 (28) to generate pRS314-57epi. Plasmid pRS314-57epi was transformed into the *RPM2* disruption strain ΔBX-6/3B and passaged on media containing 5-FOA to generate a haploid strain containing epitope-labeled *RPM2* as the only functional *RPM2* gene. The strain is phenotypically normal.

Protease digestion and sonication of mitochondria. Mitochondria were prepared from a haploid yeast bearing epitope-tagged *RPM2* by standard methods (13), frozen in liquid nitrogen, and stored at −80°C until further use. For protease digestions of mitochondria, 10 μl of mitochondria at 12.5 mg/ml was diluted 10 fold into SEH (250 mM sucrose, 1 mM EDTA, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4]) or SEH containing 0.5% Triton X-100, on ice. For mitoplast digestions, mitochondria were diluted 10 fold into buffer lacking sucrose to rupture the outer membrane. Proteinase K was added to 200 μg/ml, and reaction mixtures were incubated for 30 min on ice. Phenylmethylsulfonyl fluoride was then added to 2 mM, and the mixtures were precipitated with trichloroacetic acid (final concentration, 10%). Trichloroacetic acid-precipitated samples were solubilized in SDS sample buffer and analyzed by Western blotting (immunoblotting).

For sonication experiments, mitochondria were diluted to 0.5 mg/ml in SEH (see Fig. 6B) or SEH containing KCl at concentrations ranging from 50 mM to 1 M (not shown). One-milliliter samples were sonicated on ice by using a Kontes Micro Ultrasonic Cell Disrupter equipped with a microprobe, and six pulses of 5 s at 75% power with 15 s between pulses. Following sonication, samples were separated into supernatant and pellet fractions by centrifugation for 2 h at 100,000 × *g* at 4°C. Supernatants were trichloroacetic acid precipitated, and aliquots of both the pellet and the supernatant were analyzed by Western blotting.

Western blots were probed with the monoclonal antibody 9E10 (7) (obtained from Oncogene Science), followed by horseradish peroxidase-conjugated goat anti-mouse antisera (Bio-Rad). Development was by ECL (Amersham), and the results were quantitated by densitometry. Similar results were obtained in experiments using wild-type mitochondria and antisera to the native carboxyl terminus of Rpm2p.

RESULTS

Isolation of a high-copy suppressor of *isp42-3*. The yeast strain KKY3.3, bearing a temperature-sensitive allele of *ISP42* (*isp42-3*) was transformed with a yeast genomic library in the plasmid YEp24, which is maintained at 20 to 30 copies per cell. Transformants able to grow at the nonpermissive temperature of 35°C were selected, and the plasmids conferring the phenotype were recovered, as previously described (13). In a screen of approximately 10,000 transformants, 14 plasmids allowing growth of KKY3.3 at the nonpermissive temperature were recovered. Eleven of these were found to contain a wild-type copy of *ISP42* by PCR and restriction analysis and were not analyzed further. Of the three plasmids lacking *ISP42*, two contained *ISP6* and have been previously described (13). Analysis of the remaining plasmid, originally named 3NS57 (13), is reported here.

The 3NS57 plasmid allows the temperature-sensitive yeast strain bearing the mutant allele *isp42-3* to grow at 35°C with an efficiency similar to that of *ISP6*-containing plasmids (Fig. 1;

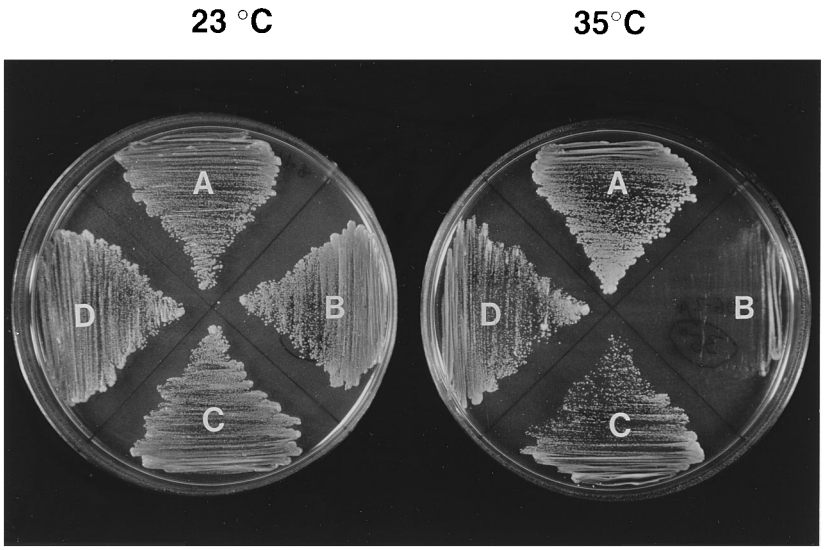

FIG. 1. 3NS57 is a high-copy suppressor of *isp42-3*. Shown are two petri dishes identically inoculated and incubated at either 23°C or 35°C, as indicated. Each sector contains the yeast strain KKY3.3 (with the temperature-sensitive *isp42-3* allele) bearing a different high-copy-number, 2μ-based plasmid. A, control plasmid with a wild-type copy of *ISP42*, which restores normal growth; B, control plasmid with no genomic insert (YEp24), showing the temperature-sensitive phenotype of KKY3.3; C, 3NS57, recovered from a yeast genomic library and seen here suppressing the temperature-sensitive growth defect; D, plasmid bearing the *ISP6* gene, which has previously been described as a high-copy suppressor of *ISP42* and is included here for comparison.

compare sectors C and D). This suggested to us that 3NS57 might also code for a component of the import apparatus. 3NS57 contains an insert of yeast genomic DNA just over 8 kb. A restriction map of this insert is shown in Fig. 2. Various subclones containing smaller genomic fragments were constructed, and the abilities of these subclones to act as high-copy suppressors of the *isp42-3* phenotype were tested. The genomic DNAs contained within these subclones and their abilities to allow growth of *isp42-3* at 35°C are diagrammed in Fig. 2.

The high-copy suppressor of *isp42-3* is *RPM2*, a gene encoding a protein subunit of mitochondrial RNase P. The genomic fragment capable of suppressing *isp42-3* was sequenced and found to contain a single gene, *RPM2*, which encodes a protein subunit of the mitochondrial RNase P enzyme (5). Complete sequence analysis of this gene revealed greater than 99% identity over 3.8 kb with the previously sequenced *RPM2* gene, including the entire open reading frame and 5′ and 3′ flanking sequences.

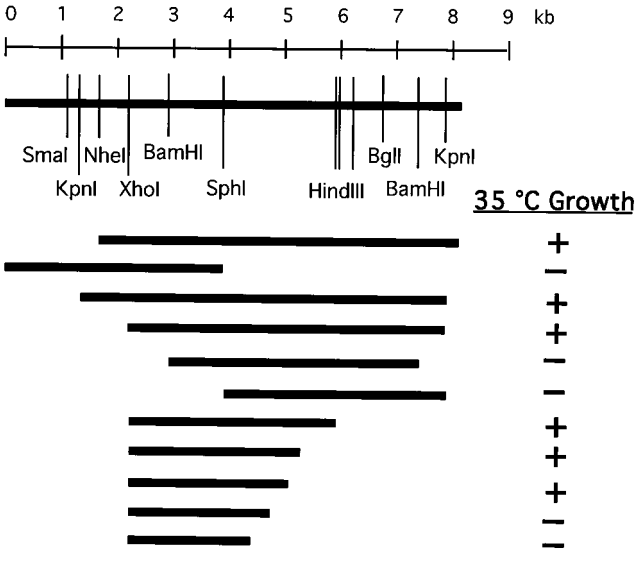

FIG. 2. *RPM2* is the gene in 3NS57 responsible for high-copy suppression of *isp42-3*. A partial restriction map of the genomic DNA insert in 3NS57 is shown. Genomic DNAs present in a set of subclones derived from 3NS57 are diagrammed below. The abilities of the subclones to act as high-copy suppressors of *isp42-3* are indicated on the right. The DNA required for suppression was sequenced and found to contain a single large open reading frame allelic to *RPM2*.

The gene isolated by suppression analysis was originally derived from DNA isolated from the yeast strain S288C, used to construct the YEp24 library (3). The *RPM2* gene isolated by screening a library for sequences that code for a protein subunit of RNase P identified by protein purification is derived from AB320 DNA used to make a YEp13 library (22). The high level of identity between the two sequences argued that the genes were allelic, and this was confirmed by high-stringency Southern blotting of yeast genomic DNA, which revealed only a single fragment homologous to these genes (not shown). Thus, the sequence differences are not significant to activity but must reflect natural polymorphisms. We will subsequently refer to the *isp42-3* suppressor as *RPM2*.

RPM2 in single copy does not suppress *isp42-3*, for the original temperature-sensitive phenotype was observed in an *RPM2* strain. In some experiments, *RPM2* on a centromere plasmid looked slightly different from plasmid alone, but it clearly does not suppress the *ISP42* temperature-sensitive phenotype effectively, whereas *RPM2* on a multiple-copy plasmid does. Regardless of plasmid copy number, *RPM2* cannot suppress a null mutation in *ISP42* (data not shown). Yeast strains PK81, containing a temperature-sensitive mitochondrial Hsp70p (*ssc1-2*) (12), and LK203, containing a temperature-sensitive Mim44p (*mim44-6*) (25) but transformed with *RPM2* on a high-copy plasmid, were not suppressed at 35 or 37°C (data not shown). Thus, *RPM2* is not a global suppressor of all mitochondrial import mutants.

The carboxyl terminus of Rpm2p is not required for suppression of *isp42-3*. The smallest genomic fragment capable of suppressing *isp42-3* (p57H7 in Fig. 2) does not contain a ter-

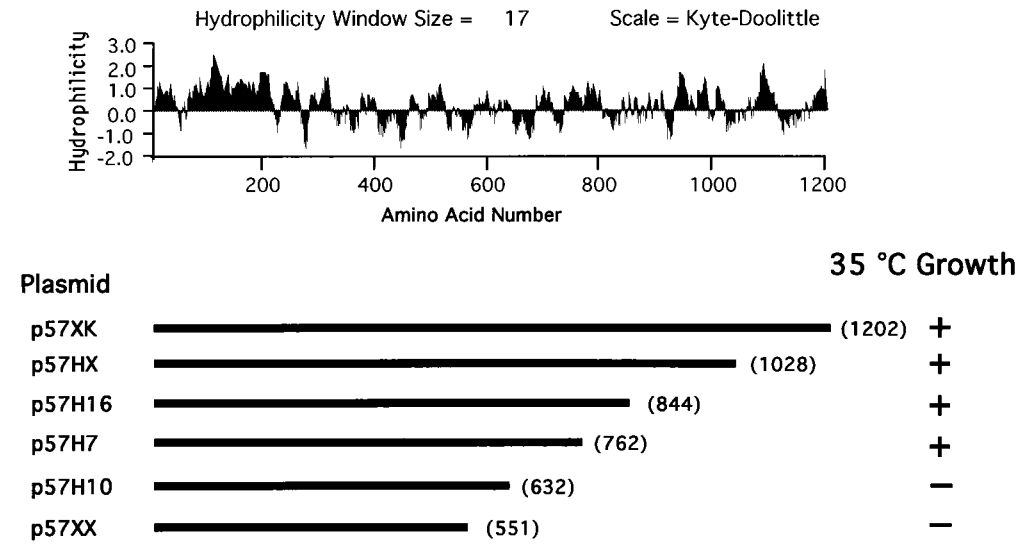

FIG. 3. Full-length RPM2p is not required for high-copy suppression of *isp42-3*. A Kyte-Doolittle hydrophilicity plot of the open reading frame of *RPM2* is shown at the top. The portions of the open reading frame present in a series of deletion constructs are diagrammed below, with the number of amino acids remaining shown in parentheses. The abilities of the constructs to act as high-copy suppressors of *isp42-3* are indicated on the right. It can be seen that as few as 762 amino acids of the 1,202-amino-acid open reading frame are sufficient for suppression.

mination codon and encodes only the amino half of *RPM2*, indicating that the carboxyl terminus of Rpm2p is not required for high-copy suppression of *isp42-3*. To better locate the region necessary for *isp42-3* complementation, a series of plasmids encoding Rpm2p with different truncations of the carboxyl terminus was constructed. The ability of each construct to complement the temperature-sensitive defect of *isp42-3* is indicated in Fig. 3, below a Kyte-Doolittle hydropathy plot (17) of the full-length reading frame encoded by *RPM2*. Deletions containing as few as 762 amino acids of the 1,202-amino-acid open reading frame are sufficient for high-copy suppression.

RNase P activity is not required for high-copy suppression of *isp42-3* by Rpm2p. Since Rpm2p containing deletions in the carboxy terminus can still suppress *isp42-3* (Fig. 3), we next asked whether the truncated constructs retained RNase P activity. This was done by constructing yeast strains carrying the *RPM2* deletion series plasmids in a background in which the genomic *RPM2* gene had been disrupted. Strains carrying a disruption of *RPM2* created by inserting the *LEU2* gene into an *Hpa*I site at the position coding for amino acid 735 are compromised in mitochondrial RNase P activity. Each carboxyl terminal deletion plasmid was transformed into this disruption strain to determine which plasmid(s) could complement the mitochondrial RNase P defect. The resulting transformants were scored for their ability to process mitochondrial tRNAs by isolating and separating RNAs on gels, transferring the RNA to nylon membranes, and probing with an RNA complementary to mitochondrial $tRNA_f^{Met}$. In Fig. 4 it can be seen that mature tRNA is present in RNA prepared from the wild-type yeast (lane 1) and that there is no mitochondrial $tRNA_f^{Met}$ detected in *rho*^{0} strains which contain no mitochondrial DNA (lane 2). Yeasts carrying the disruption but no plasmid-encoded *RPM2* accumulate precursor tRNA (lane 3), and the disruption strain transformed with a plasmid encoding wild-type Rpm2p recovers the ability to process the tRNA precursor to mature tRNA (lane 4). Plasmids producing Rpm2p with increasing deletions at the carboxyl terminus are shown in lanes 5 to 10. Precursor tRNAs are present in all strains transformed with *RPM2* truncations (Fig. 4; compare lanes 5 to 10 with lane 1). Deletions of as few as 174 amino acids lead to a loss of detectable mitochondrial RNase P activity in these cells, whereas deletion of even 440 amino acids still allows full complementation of *isp42-3* (Fig. 3). Since the carboxyl terminus is not required for suppression of *isp42-3* but is necessary for maintenance of RNase P activity under these growth conditions, we conclude that RNase P activity is not required for suppression of *isp42-3*.

***RPM2* is essential for normal cell growth.** The first *RPM2* mutation described was an insertion of the *LEU2* gene into an *Hpa*I site corresponding to amino acid 735 of the 1,202-amino-acid open reading frame of *RPM2* (20). Like many other mutations leading to severe defects in mitochondrial protein synthesis, this disruption led to the development of petite deletion mutants, as maintenance of the wild-type mitochondrial genome seems to depend on protein synthesis in the organelle (21). Once the mitochondrial DNA is lost, cells are unable to grow on the nonfermentable carbon source, glycerol, because loss of mitochondrial DNA causes defects in respiration. This initial genetic analysis of *RPM2* gave the expected phenotype

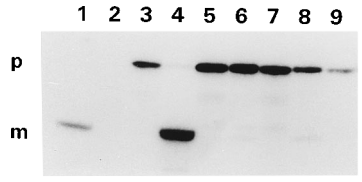

FIG. 4. C-terminal deletions cause defects in RNase P activity. A Northern blot containing total RNAs from different yeast strains and probed with a $tRNA_f^{Met}$-specific probe is shown. p and m, precursor and mature $tRNA_f^{Met}$, respectively. Lane 1, RNA from the wild-type yeast; lane 2, RNA from a rho^0 strain lacking mitochondrial DNA; lane 3, RNA from the *RPM2* disruptant strain (*rpm2*::*LEU2*) which lacks RNase P activity (5); lane 4, RNA from the *rpm2*::*LEU2* strain bearing the plasmid p57XK (containing a full-length *RPM2* gene), which restores RNase P activity; lanes 5 to 9, RNAs from *rpm2*::*LEU2* strains bearing plasmids with increasing truncations of *RPM2*—p57HX, p57H16, p57H7, p57H10, and p57XX, respectively—which appear to be unable to restore RNase P activity.

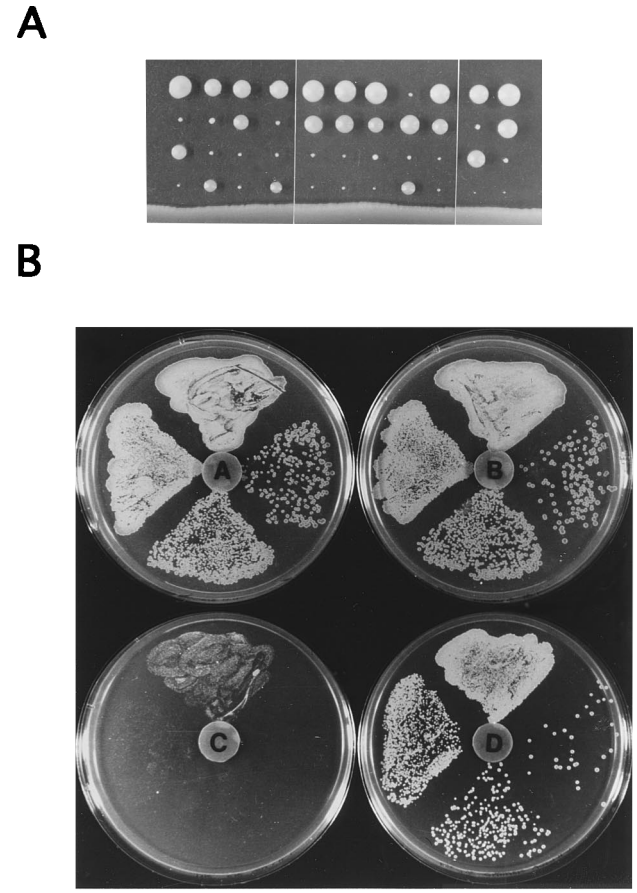

FIG. 5. Phenotype of *rpm2* null mutants. (A) Diploid cells heterozygous for an insertional disruption of *RPM2* by *HIS3* at the *Sph*I site were sporulated, and tetrads were dissected. The large colonies showing wild-type growth are His^-, but the few cells that do grow upon streaking of the small colonies to fresh media are His^+. (B) *rpm2* null mutants are not recovered on synthetic media. Petri dishes containing haploid yeast cells with a deleted chromosomal *RPM2* gene and a wild-type copy of *RPM2* on an exogenously replicating plasmid bearing the *URA3* gene as a selectable marker (BXLeu2/RPM2 cells; see Materials and Methods for strain construction) are shown. In addition, the cells contain a second plasmid with *HIS3* as the selectable marker and either a second wild-type copy of *RPM2* (plates B and D) or no insert (plates A and C). Each plate contains four sectors that are serial 10-fold dilutions of the same yeast transformant. Plates A and B are synthetic media, and plates C and D are synthetic media containing 5-FOA. Only cells which have lost the *URA3* plasmid are able to grow on 5-FOA media. Cells whose only copy of *RPM2* is on the *URA3* plasmid are unable to grow on 5-FOA media (compare plates A and C), whereas cells with *RPM2* also provided on the *HIS3* plasmid readily lose the *URA3* plasmid and are able to grow on 5-FOA (compare plates B and D).

and did not suggest that *RPM2* was necessary for anything other than mitochondrial RNase P activity.

To further investigate the phenotype caused by loss of *RPM2*, we constructed additional yeast strains in which the coding sequence of *RPM2* was disrupted by insertion of a marker gene at a different site or in which large regions of the coding sequence of *RPM2* were deleted. In contrast to the petite phenotype observed with the insertional disruption of *RPM2* at the *Hpa*I site, we find that cells with an *RPM2* disruption closer to the amino terminus (at the *Sph*I site, corresponding to amino acid 362 in the 1,202-amino-acid open reading frame) have a much more severe phenotype. Fig. 5A shows a dissection of tetrads obtained following sporulation of diploid cells heterozygous for an insertion of *HIS3* into the *Sph*I site of *RPM2*. Each tetrad yielded two wild-type colonies, which lacked the *HIS3* marker, and two very small (diameter, less than 1 mm) colonies containing the *RPM2* disruption. The very small colonies soon ceased growing on the original plates and did not continue normal growth when picked and restreaked on fresh rich plates.

After prolonged incubation (over a week), the restreaked colonies gave rise to multiple subcolonies which grew at variable (slow) rates on glucose and were unable to grow on the nonfermentable substrate glycerol. An identical phenotype was observed following sporulation of diploid cells heterozygous for large deletions in *RPM2*, including a complete and precise deletion of the entire coding sequence of *RPM2* (not shown). This pattern of behavior suggests that the gene is essential for normal growth but other genetic events or extragenic suppressors can restore viability to a subset of cells.

As an alternative way to generate cells lacking the *RPM2* gene, we performed a plasmid loss experiment (Fig. 5B). A haploid strain containing a deletion in the genomic *RPM2* gene but containing a wild-type copy of *RPM2* on a centromere plasmid which also contains the *URA3* gene as a selectable marker was constructed (see Materials and Methods for details of strain construction). These phenotypically normal cells were then transformed with a second plasmid carrying *HIS3* as a selectable marker and additionally containing either wild-type *RPM2* or no insert. Cells containing the two plasmids were then plated onto synthetic media containing 5-FOA, which selects for cells that have lost the *URA3*-containing plasmid (*URA3*-bearing cells convert 5-FOA into the toxic metabolite 5-fluorouracil). Cells in which the *HIS3* plasmid contains wild-type *RPM2* can readily lose the *URA3* plasmid and grow well on 5-FOA (Fig. 5B, plate D). In contrast, when the *HIS3* plasmid lacks *RPM2*, loss of the *URA3* plasmid leaves the cells without any functional copy of *RPM2*, and such cells are seen to be inviable (Fig. 5B; compare plate C with plate D). This experiment demonstrates that *RPM2* is essential in the W3031A/B background on synthetic media. Additional experiments also support this conclusion. For example, repeated attempts to create deletion disruptions of *RPM2* by directly transforming haploid W3031a cells have been unsuccessful.

***RPM2* sequences that suppress *isp42-3* also provide the function essential for normal growth.** We transformed each of the *RPM2* C-terminal deletion plasmids into diploid cells containing one deleted copy and one wild-type copy of *RPM2*. The transformants were then sporulated and dissected to assess the phenotype of the truncated *RPM2* products.

When diploids contained *RPM2* plasmids capable of suppressing *isp42-3* (p57XK, p57HX, p57H16, and p57H7 [Fig. 3]), all four spores appeared phenotypically normal on rich glucose media. As expected, however, subculture on glycerol revealed that the two spores containing the chromosomal deletion of *RPM2* and a truncated plasmid-borne copy of *RPM2* had sustained deletions in their mitochondrial DNAs and had become respiration deficient. The two spores with a wild-type chromosomal copy of *RPM2* retained their mitochondrial DNAs (data not shown).

When diploids contained *RPM2* plasmids that are not capable of suppressing *isp42-3* (p57H10 and p57XX), a different pattern was observed upon sporulation. On rich glucose media, only two spores were phenotypically normal, and the other two spores germinated but failed to continue to grow (data not shown). The plasmids that cannot suppress the *isp42-3* temperature-sensitive allele were thus unable to complement the growth defect caused by the chromosomal deletion of *RPM2*, and we conclude that suppression of the *isp42-3* allele and the function essential for normal growth are provided by the same region of *RPM2*.

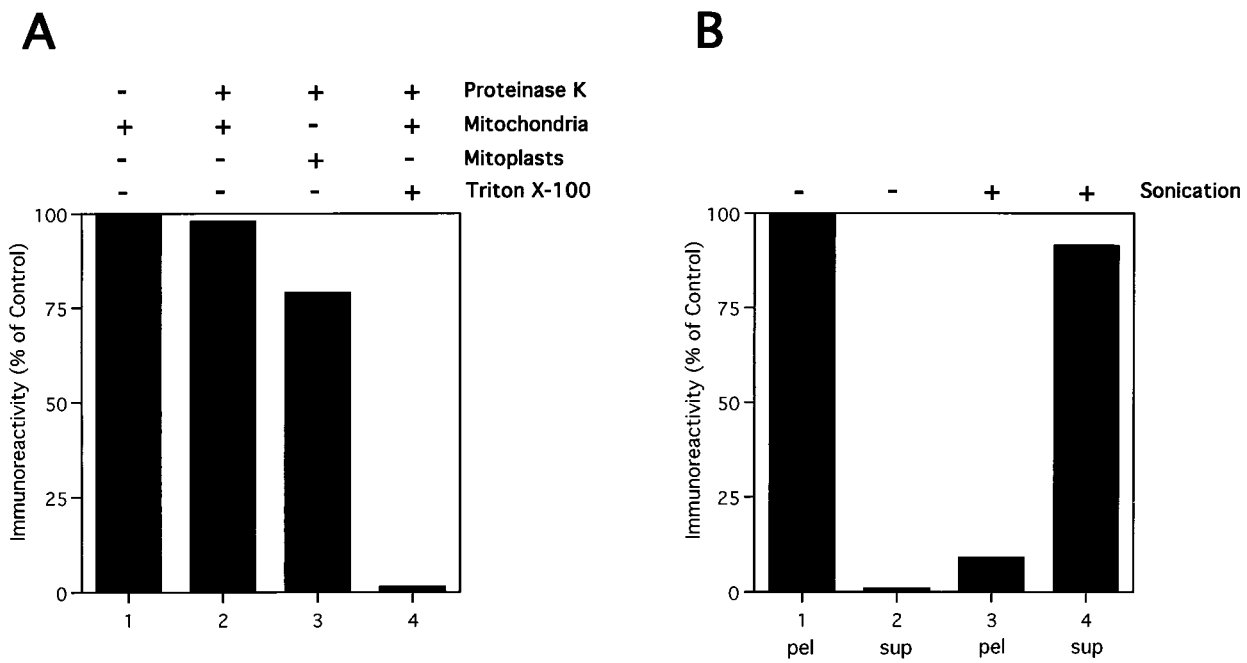

FIG. 6. Rpm2p is a soluble protein of the mitochondrial matrix. (A) Rpm2p resists protease digestion in intact mitochondria and in mitoplasts. Mitochondria (lanes 2 and 4) or mitoplasts (lane 3) were digested with 200 μg of proteinase K per ml for 30 min on ice as described in Materials and Methods. Phenylmethylsulfonyl fluoride was then added to inactivate the protease, the reaction mixtures were precipitated with trichloroacetic acid, solubilized in SDS sample buffer, and analyzed by Western blotting. It can be seen that Rpm2p is largely resistant to protease digestion in the absence of detergent. Mitoplast formation under these conditions is essentially complete, as measured by digestion of the intermembrane space protein cytochrome b_2 (not shown). (B) Rpm2p is released from mitochondria by sonication. Mitochondria were sonicated on ice as described in Materials and Methods, and then soluble and membrane-associated fractions were separated by centrifugation for 2 h at 100,000 × *g*. Aliquots of the pellet (pel) and supernatant (sup) were solubilized in SDS sample buffer and analyzed by Western blotting. The vast majority of Rpm2p immunoreactivity is recovered in the soluble fraction after sonication in low-salt buffer.

Rpm2p is a soluble protein of the mitochondrial matrix. Rpm2p was first identified as the major protein that copurifies with the RNase P activity of yeast mitochondria (20). Published procedures for the isolation of Rpm2p have used detergent and a high salt concentration to solubilize the enzyme from mitochondria, but its intramitochondrial location has not been examined. Rpm2p has an established role in mitochondrial RNA metabolism, which presumably occurs in the mitochondrial matrix. As we show here, Rpm2p also suppresses mutations of the outer mitochondrial membrane protein, ISP42p. If this suppression is due to physical interaction between the two proteins, Rpm2p might be expected to be a transmembrane protein, with domains on both sides of the inner mitochondrial membrane. Examination of the Kyte-Doolittle hydrophilicity plot (17) shown in Fig. 3 reveals several small hydrophobic regions, yet none are strongly predicted to be membrane-spanning domains, and the program ALOM (15) predicts that the protein is not an integral membrane protein.

To determine the submitochondrial location of Rpm2p, we performed several experiments. Figure 6A shows that the protein is resistant to externally added protease both in intact mitochondria (compare lanes 1 and 2) and in mitoplasts in which the outer membrane has been ruptured by osmotic shock (lane 3). Conversion to mitoplasts is complete under these conditions, as measured by complete digestion of the intermembrane space protein cytochrome b_2 (not shown). Rpm2p is readily digested by protease only when detergent is added to disrupt both membranes (lane 4), a finding consistent with localization to the matrix. It should be noted, however, that some nonmatrix proteins are resistant to protease digestion in the absence of detergent (for example, Isp42p behaves in this way [10]). To determine whether Rpm2p is really located in the matrix or is an inner membrane protein resistant to protease treatment, mitochondria were disrupted by sonication and separated into soluble and membrane fractions by centrifugation (Fig. 6B). Sonication of mitochondria releases the vast majority of Rpm2p into the soluble fraction under all salt conditions tested, including a very low salt concentration, indicating that Rpm2p is not a membrane protein. As a control, immunoreactive Isp42p, an integral outer membrane protein, is completely recovered in the pellet in these conditions (not shown). Taken together, the data indicate that the majority of Rpm2p is a soluble matrix protein. This finding makes it difficult to explain the genetic suppression observed in terms of a simple model of direct physical interaction between the two proteins. However, the total amount of Rpm2p in cells is very low, and thus, a small percentage of Rpm2p located in another compartment might be missed. We therefore cannot rule out the possibility that some Rpm2p might be localized outside the matrix, even though we cannot detect it. If a small percentage of Rpm2p is located outside the mitochondrial matrix, direct physical interaction of the two proteins could occur and could explain the observed suppression. Alternative models will be discussed below.

DISCUSSION

We describe here the identification of *RPM2* as a high-copy suppressor of *isp42-3*, an allele of a mitochondrial protein translocation channel component that results in temperature-sensitive growth. This finding is quite surprising, since Rpm2p was previously described as a protein subunit of mitochondrial RNase P, an enzyme not expected to play a role in the trans-

location of proteins into mitochondria. However, we show here that the suppression of *isp42-3* by *RPM2* is not related to RNase P activity, since C-terminal truncation mutants of *RPM2* lacking detectable RNase P activity are still able to complement *isp42-3*.

This result suggested to us that Rpm2p might be a bifunctional (or multifunctional) protein and that a second, unidentified activity might be responsible for suppression of *isp42-3*. Additional support is lent to this hypothesis by analysis of the null phenotype of *RPM2*. An insertional disruption mutant of *RPM2* that compromised mitochondrial RNase P activity and led to the production of petite mutants has been previously reported (20). We show here that insertional disruption of *RPM2* at a site closer to the amino terminus of the protein, or complete deletion of the *RPM2* open reading frame, yields a much more severe phenotype. Spores bearing complete coding-sequence deletions of *RPM2* are able to germinate and grow for several rounds of cell division, but then growth ceases. When the tiny colonies bearing the null allele are replated on rich media, they do not resume normal growth. Over time, a small fraction of the cells give rise to viable colonies, which grow at different (although slow) rates on glucose and are unable to grow on nonfermentable carbon sources. The very low percentage of cells giving rise to such colonies, and the long lag before their appearance, suggests that the gene is essential and that these colonies are extragenic suppression mutants. Furthermore, when null mutants in *RPM2* are generated by plasmid loss on 5-FOA media rather then by sporulation, we find that they are not viable on synthetic media.

In contrast to these findings, null mutations in *RPM1*, the mitochondrial gene encoding the RNA component of mitochondrial RNase P, also abolish RNase P activity but have no effect on growth on fermentable carbon sources (19). Furthermore, petite mutants completely lacking all mitochondrial DNA (including all mitochondrial tRNA genes) are viable. Thus, *RPM2* must have another, more essential function, separable from its role in tRNA processing.

The data we present here raise two important questions which we are not yet able to answer: what is the second function of *RPM2*, and by what mechanism does it act as a high-copy suppressor of *isp42-3*? Several possibilities exist. One is that *RPM2* codes for a second product essential for normal growth. An examination of *RPM2* in all six reading frames reveals several short open reading frames, but only one falls in the region known to be essential. A frameshift mutation that shifts the Rpm2p frame but not the short open reading frame abolishes the function essential for normal growth and shows that Rpm2p, or a peptide derived from it, supports the second function (8a). The severe cell growth defect in *RPM2* null mutants and the ability of *RPM2* to suppress a mutant allele of the protein import channel component, *ISP42*, suggest the possibility that Rpm2p plays a direct role in mitochondrial protein import. *ISP42*, for example, is essential for normal growth because of its central role in the biogenesis of an essential organelle (2). The simplest model to explain high-copy suppression of *ISP42* by *RPM2* is that the two gene products physically interact, and that increased amounts of Rpm2p stabilize the temperature-sensitive *isp42-3* allele. Our data showing that most Rpm2p is a soluble matrix protein would appear to preclude such a direct physical interaction with the majority of Rpm2p (except during the import of Rpm2p). Nonetheless, we cannot rule out the possibility that a small fraction of Rpm2p might be localized to the intermembrane space or the cytoplasm, where such an interaction could occur. We have attempted to find evidence for a direct interaction between Isp42p and Rpm2p by co-immune precipitation studies and chemical cross-linking, but efforts to date have been unsuccessful (12a).

Even as a soluble matrix protein, however, Rpm2p might still be involved in the import of proteins into mitochondria. For example, the mitochondrial form of Hsp70p (encoded by the *SSC1* gene) is a soluble matrix protein that plays an essential role in import of proteins into the mitochondrial matrix (12). Similarly, mitochondrial Hsp60p (encoded by *MIF4*) is a soluble matrix protein that is essential for correct folding and assembly of matrix proteins (4). One possible mechanism to explain high-copy suppression of the outer membrane protein Isp42p by a matrix protein could be that the effect is mediated through a third component. For example, protein import channels in the outer membrane interact dynamically with import channels in the inner membrane (8, 11, 24). Perhaps, Rpm2p binds to the matrix side of the inner membrane channel when it is engaged with the outer membrane. Such binding could act to stabilize the connection between the channels, locking them into place. Overexpression of Rpm2p might then help to drive the formation of such channel couplings in the isp42-3 mutant, whose altered channels might interact less stably. With regard to this model it is notable that a portion of Hsp70p has recently been shown to be reversibly bound to the inner membrane and to interact directly with Mim44p, a component of the mitochondrial inner membrane protein translocation apparatus (25). Thus, a portion of Rpm2p might behave in a similar fashion.

Preliminary efforts to confirm a direct involvement of *RPM2* in mitochondrial protein import have been unsuccessful. For example, attempts to cross-link Rpm2p to precursors undergoing mitochondrial import, or to other members of the mitochondrial import machinery, have so far been unfruitful (12a). We have also examined several of the *RPM2* truncation mutants for evidence of mitochondrial protein precursor accumulation but have not seen it in the mutants examined thus far (12a). In addition, we have constructed yeast strains in which expression of *RPM2* is under the control of the *GAL10* promoter and had hoped to examine the cells for mitochondrial precursor accumulation when *RPM2* expression is repressed by growth in glucose. However, to date our galactose-dependent constructions are somewhat leaky, and even the very low levels of transcription obtained from the *GAL10* promoter during growth in glucose provide sufficient Rpm2p for complete phenotypic normality. These negative findings do not rule out a role for *RPM2* in mitochondrial protein import, and further studies are ongoing. We are currently working to generate temperature-sensitive alleles of *RPM2*, which could be examined at the nonpermissive temperature for clues as to the second function of this gene.

If the second function of *RPM2* is not directly involved in mitochondrial protein import, what might its function be, and how could it act as a high-copy suppressor of *isp42-3*? We have shown here that *RPM2* (but not mitochondrial RNase P activity) is essential for normal yeast growth. Mitochondria are the site of many essential cellular processes including gluconeogenesis, heme biosynthesis, amino acid biosynthesis, fatty acid metabolism, etc., and Rpm2p might conceivably be involved in any of these processes in addition to its role in RNA processing. A decreased level of mitochondrial Rpm2p caused by the isp42-3 mutation could compromise a putative second function in any of these processes and be largely responsible for the lack of growth of the mutant at 35°C. This hypothesis requires either that the import of Rpm2p be more severely affected than other mitochondrial proteins by the adverse import conditions at 35°C or, alternatively, that import of all proteins into mitochondria be reduced at 35°C but cells be particularly sen-

sitive to a reduction in the levels of Rpm2p. If the amount of Rpm2p in the cell is increased (by higher gene dosage), more Rpm2p might reach the matrix and allow growth at 35°C. At even higher temperatures (e.g., 37°C), import of mitochondrial proteins would be so impaired that the high copy number of *RPM2* is no longer able to restore growth. Of course, other models could also be imagined.

Admittedly, our current knowledge of *RPM2* is incomplete, and we have neither ruled out a role for *RPM2* in mitochondrial protein import nor provided evidence to support other possible explanations discussed above. We have shown that *RPM2* has a function required for normal growth which appears separate from the role it plays in mitochondrial tRNA processing. The finding that *RPM2* is a suppressor of *isp42-3* is intriguing, but the mechanism is as yet unclear. We will continue to apply the powerful tools of yeast genetics, molecular biology, and biochemistry to further elucidate this other function of *RPM2* and to understand the interrelationships suggested by the results presented here.

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