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

C. KENNETH KASSEN BroCK,†‡ GUO-JIAN GAO,‡ KATHLEEN R. GROOM,‡ PAVOL SULO,‡‡ MICHAEL G. DOUGLAS,§ AND NANCY C. MARTIN*a

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, Kentucky2

Received 8 March 1995/Returned for modification 11 April 1995/Accepted 1 June 1995

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 (15). 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.
Northern (RNA) analysis was performed as described previously (20) by using a probe made from pPM6 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 isp2-3 was termed 3NS57. 3NS57 was cut with XhoI and KpnI, which had been digested with EcoRI, and a 5.67-kb fragment containing the HIS3 gene was subcloned into the vector p306-2. The insert was then excised from this vector by using XhoI and EcoRI and subcloned into p306-2m cut with the same enzymes. Plasmids p57H7, p57H10, and p57H16 were derived from p57H7 as follows. p57H7 was cut with Smal and SacI and digested with exonuclease III by standard methods (26). SacI was then used to digest one of the fragments at the appropriate time points during the treatment were reacted with mung bean exonuclease and then ligated to generate a series of nested deletions extending in from the HindIII side of the insert. Plasmid p57XX consists of a 2.54-kb XhoI-XbaI fragment from 3NS57 cloned into p306-2m and was made by cutting pBS-57H7 with XhoI, religating, and then cutting the insert out of the resulting plasmid with XhoI and EcoRI and subcloning into p306-2m cut with the same enzymes.

Gene disruptions. The disruption of RPM2 at the Hpy1 site has been described previously (20). An insertional disruption of RPM2 at the Spel site made as follows. pBS-57H7 was digested with Spel, and the resulting ends were filled in by the Klenow enzyme. A 1.3-kb BamHI-XhoI fragment containing the HIS3 gene was made blunt by the Klenow enzyme and ligated to the above to generate p57HI. p57HI was cut with XhoI and HindIII, and the three fragments were ligated together. Removal of the sequence coding for amino acid 144 to 177 of RPM2 was accomplished by cutting this plasmid with XhoI and XbaI; ends were filled in by the Klenow enzyme and a 2.2-kb HpaI fragment containing the LEU2 gene from Yep13 was inserted. The resultant plasmid, Yep352/BXLUE2, was digested with BamHI and a 3.6-kb fragment containing the gene deletion was introduced into the diploid yeast W303 by cotransformation. 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 spored, tetrads were dissected, and Ura+ spores were selected to generate haploid BXLUE2/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. A primer, which cuts once in the insert 24 bp downstream of the XhoI site, was designed (AGCAAGCCTCTTCAAGCAGAACAAAAGCTTATTTCTGAAGAGCG, (GTAACACGCGACCGCAGAT) and 3’-B-57-388 (CGCGCATTCCTTTCGGTGTATGCTTGTT)). The product of this reaction was cut with XhoI and BamHI and subcloned into pSP72 (Promega). A second PCR was performed with the first two primers and primers 5’-GCTGGTACACATTAATATATTTTATT and 57-3’-Kpn (described above). The 502-bp product was gel-purified and designated product 1. A third PCR was performed with p57XK as template and primers 57-CEPI-5 (AGCAAGCCTCTTCAAGCAGAACAAAAGCTTATTTCTGAAGAGCG) and 5’-CTAGTGATCCATGTCTCAGGACGCTGTCGCTCAGGCGTGG) 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 (TTGAACCCCTTACTCCTCCTTCT) and 57-CEPI-3’ (ATTAATTGTTAAG GTCAACAGTCTTCGCGAAATAGAGCTTGCTGCTGAAAGGCTTG). The 264-bp product was cut with the same enzymes. A second PCR was performed with p57XK as template and primers 57-CEPI-5’ (AGCAAGCCTCTTCAAGCAGAACAAAAGCTTATTTCTGAAGAGCG) and 5’-CTAGTGATCCATGTCTCAGGACGCTGTCGCTCAGGCGTGG) 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 XhoI and KpnI and ligated to the 10.7-kb band resulting from cutting p57XK with KpnI and partially digesting with XbaI. The resultant plasmid is termed p306-2m-57epi. The DNA sequence from the XhoI site downstream of the coding sequence was confirmed to be correct by DNA sequencing. This plasmid was cut with XhoI and KpnI, and the insert was subcloned into pRS314 (28) to generate pRS314-57epi. Plasmid pRS114-57epi was transformed into the RPM2 disruption strain ΔBSX-3/8 and passed 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.

Preparation and digestion 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 probe digestion 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 7.5]) or SEH containing 0.5% Triton X-100, on ice. For mitoplast digestion, mitochondria were diluted 10 fold into buffer lacking sucrose to rupture the outer membrane. Protease K was added to 200 µg/ml and reaction mixture was 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 10,000 × g at 4°C. Supernatants were trichloroacetic acid precipitated, and pellets 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 IgG (Bio-Rad Laboratories). The results were quantitated by densitometry. Similar results were obtained in experiments using wild-type mitochondria and antisera to the native carboxyl terminus of RPM2.

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 described previously (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. None 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;
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.

The gene isolated by suppression analysis was originally derived from DNA isolated from the yeast strain S288C, used to construct the YEpl4 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 YEpl3 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 (scc1-2) (12), and LK203, containing a temperature-sensitive Min44p (min44-6) (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. 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 (YEpl4), 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 DNPH gene, which has previously been described as a high-copy suppressor of ISP42 and is included here for comparison.
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 hydrophathy 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 HpaI 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. 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\textsubscript{Met}. In Fig. 4 it can be seen that mature tRNA\textsubscript{Met} is present in RNA prepared from the wild-type yeast (lane 1) and that there is no mitochondrial tRNA\textsubscript{Met} detected in rho\textsuperscript{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 HpaI 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.
but the few cells that do grow upon streaking of the small colonies to fresh media containing 5-FOA. Only cells which have lost the Plasmid RPM2 copy of a wild-type copy of RPM2 on an exogenously replicating plasmid bearing the URA3 gene as a selectable marker (BXLeu2-2 plasmids; 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 on an exogenously replicating plasmid bearing the URA3 gene or a truncated plasmid-borned copy of RPM2 (p57H10 and p57XX), a different 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 (p57XX, 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-borned 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.
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 mitochondriom 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 *SSCI* 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 Mif44p, 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.

REFERENCES