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Rpm2, the Protein Subunit of Mitochondrial RNase P in *Saccharomyces cerevisiae*, Also Has a Role in the Translation of Mitochondrially Encoded Subunits of Cytochrome c Oxidase

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ABSTRACT

RPM2 is a *Saccharomyces cerevisiae* nuclear gene that encodes the protein subunit of mitochondrial RNase P and has an unknown function essential for fermentative growth. Cells lacking mitochondrial RNase P cannot respire and accumulate lesions in their mitochondrial DNA. The effects of a new *RPM2* allele, *rpm2-100*, reveal a novel function of *RPM2* in mitochondrial biogenesis. Cells with *rpm2-100* as their only source of Rpm2p have correctly processed mitochondrial tRNAs but are still respiratory deficient. Mitochondrial mRNA and rRNA levels are reduced in *rpm2-100* cells compared to wild type. The general reduction in mRNA is not reflected in a similar reduction in mitochondrial protein synthesis. Incorporation of labeled precursors into mitochondrially encoded Atp6, Atp8, Atp9, and Cytb protein was enhanced in the mutant relative to wild type, while incorporation into Cox1p, Cox2p, Cox3p, and Var1p was reduced. Pulse-chase analysis of mitochondrial translation revealed decreased rates of translation of COX1, COX2, and COX3 mRNAs. This decrease leads to low steady-state levels of Cox1p, Cox2p, and Cox3p, loss of visible spectra of aa_3 cytochromes, and low cytochrome c oxidase activity in mutant mitochondria. Thus, *RPM2* has a previously unrecognized role in mitochondrial biogenesis, in addition to its role as a subunit of mitochondrial RNase P. Moreover, there is a synthetic lethal interaction between the disruption of this novel respiratory function and the loss of wild-type mtDNA. This synthetic interaction explains why a complete deletion of *RPM2* is lethal.

MITOCHONDRIAL DNA (mtDNA) in the yeast *Saccharomyces cerevisiae* codes for components of complexes required in oxidative phosphorylation and electron transport as well as RNAs necessary for their expression by the mitochondrial translational machinery (Attardi and Schatz 1988; Pon and Schatz 1991). The vast majority of mitochondrial proteins, however, are encoded by nuclear genes, translated on cytoplasmic ribosomes, and delivered to mitochondria for function (Grivell *et al.* 1999).

S. cerevisiae has been a useful organism for studying many aspects of mitochondrial biogenesis because, as a facultative anaerobe, it can grow by either fermentation or respiration. Therefore, depending on the carbon source used, mutants with defects in genes required for respiration can be recovered and studied. A problem associated with using yeast to study aspects of mitochondrial gene expression is the link between translation of mitochondrial gene products and the maintenance of the mitochondrial genome. Mutations in nuclear genes that disrupt mitochondrial gene expression induce either the complete loss of the mitochondrial genome or large fragments thereof (Myers *et al.* 1985). Secondary effects associated with the loss of the mitochondrial genome can be particularly troublesome in the study of multifunctional proteins when one function is required for mitochondrial gene expression. A case in point is the nuclear gene, *RPM2*, which encodes a protein subunit of mitochondrial RNase P (Morales *et al.* 1992; Dang and Martin 1993). The RNA subunit of mitochondrial RNase P, Rpm1r, is encoded in the organelle (Miller and Martin 1983; Underbrink-Lyon *et al.* 1983). Mitochondrial RNase P is an enzyme required for processing 5′ leader sequences from organelle tRNAs. Cells carrying an insertional disruption of *RPM2* (*rpm2::LEU2*) produce a carboxyl-terminally truncated Rpm2p and accumulate mitochondrial tRNA precursors with extensions at their 5′ ends (Morales *et al.* 1992). Like other nuclear and mitochondrial mutants defective in mitochondrial protein synthesis (Myers *et al.* 1985), these cells cannot maintain their mitochondrial DNA and either accumulate mitochondrial genomes with large deletions or lose their mitochondrial genomes entirely.

The unexpected observation that a complete deletion of the *RPM2* open reading frame prevented growth on fermentable carbon sources revealed that Rpm2p has another function, in addition to its role in RNase P activity (Kassenbrock *et al.* 1995). This second function

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could be involved in mitochondrial biogenesis and yet still affect growth on fermentable carbon sources. For example, mutations in genes encoding components of the mitochondrial import machinery affect the localization of proteins required for a number of essential functions in addition to proteins required for respiration (Baker and Schatz 1991; Herrmann and Neupert 2000). Alternatively, it has been shown that certain proteins required for respiratory growth become essential for growth on fermentable carbon sources in the absence of a wild-type mitochondrial genome (for review see Chen and Clark-Walker 1999b; Contamine and Picard 2000). However, a mechanism for this phenomenon has not been established.

Although we have previously isolated alleles of *RPM2* that lose mitochondrial RNase P activity, but allow cells to grow on fermentable carbon sources, we had not isolated any that retain RNase P activity but were otherwise compromised for growth. Here we describe a novel allele of *RPM2*, *rpm2-100*, which produces a protein missing amino acids 146–246. Cells with this allele as their sole source of Rpm2p grow on fermentable media, retain mitochondrial RNase P activity *in vivo*, and maintain wild-type mitochondrial genomes. However, even though mitochondrial tRNA processing appears normal, *rpm2-100* cells grow poorly on respiratory carbon sources. Further analysis revealed that these cells have a specific defect in the synthesis of mitochondrially encoded cytochrome c oxidase subunits. This results in the loss of visible spectra of aa_3 cytochromes and low cytochrome c oxidase activity. Therefore, Rpm2p has another mitochondrial function, in addition to its role in RNase P activity. Interestingly, cells defective in this second function possess a limited capacity to divide upon losing the wild-type mitochondrial genome. These data are consistent with the view that *RPM2* has two mitochondrial functions and the loss of either affects growth on nonfermentable carbon sources. Moreover, mutations that disrupt both functions are synthetically lethal.

MATERIALS AND METHODS

Strains and media: Strains used in this study are listed in Table 1. Rich glucose media, YPD, included 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. Rich glycerol/ethanol media, YPGE, contained 1% Bacto-yeast extract, 2% Bacto-peptone, 3% (v/v) glycerol, and 2% (v/v) ethanol. Synthetic complete (SC) media lacking specific amino acids contained 0.67% Bacto-yeast nitrogen base, 2% glucose. Solid media for plates included 2% Bacto-agar. Culture media reagents were Fisher Scientific (Pittsburgh) or Difco (Detroit) brand. Standard yeast methods (Sherman *et al.* 1986) were used. Yeast cells were transformed with plasmid DNA using the lithium acetate method (Chen *et al.* 1992). The plasmid-shuffle protocol was performed as described (Sikorski and Boeke 1991).

Construction of *RPM2* mutants: Standard procedures were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *Escherichia coli* (Sambrook *et al.* 1989). Restriction and modification enzymes were used as recommended by the supplier (New England Biolabs, Beverly, MA). Plasmid DNA was isolated using QIAGEN columns, and DNA fragments were isolated from agarose gels using a QIAEX II extraction kit (QIAGEN, Chatsworth, CA). DNA was sequenced with a Sequenase Version 2.0 Kit (United States Biochemical, Cleveland). To construct plasmid pRS314/*rpm2-100*, plasmid pRS315/*GAL-COX4-247.rpm2* (containing *RPM2* sequence coding for amino acids 247–1202 fused to the *COX4* mitochondrial targeting sequence under the transcriptional control of the *GAL1* promoter; our laboratory, unpublished data) was cut with *Eco*RV, which cuts downstream of the *COX4* sequence, and *Sac*I, which cuts downstream of the *RPM2* gene. Plasmid pRS314/*RPM2* containing wild-type *RPM2* was cut with *Bgl*II, filled in with Klenow, and subsequently cut with *Sac*I. After gel electrophoresis, a 5780-bp fragment containing the plasmid sequence with 5′-end sequence (−570 to +432) of *RPM2* was ligated with the *Eco*RV/*Sac*I fragment. This pRS314/*rpm2-100* construct resulted in a deletion of amino acids 146–246 coding region. To construct plasmids pRS425/*RPM2* and pRS425/*rpm2-100*, *Xho*I/*Sac*I fragments containing *RPM2* and *rpm2-100* gene were cloned into pRS425 cut with *Xho*I/*Sac*I. To integrate the *rpm2-100* allele at the *RPM2* locus, pRS314/*rpm2-100* was cut with *Xho*I and *Sac*I and a 4.0-kb fragment containing *rpm2-100* was cloned into pRS316. Integration of *rpm2-100* at the *RPM2* locus was performed as described (Rothstein 1991) and confirmed by PCR. To construct Δ735-1190, pRS314/*RPM2* was cut with *Hpa*I/*Ppu*MI, filled in with Klenow, and ligated. To construct Δ715-1098, pRS314/*RPM2* was cut with *Pst*I, gel purified, and ligated. To construct Δ528-734, pRS314/*RPM2* was cut with *Hinc*II, gel purified, and ligated.

DNA and RNA analysis: Total yeast RNA was isolated by hot phenol extraction (Kohrer and Domdey 1991). Total RNA (25–30 μg) was separated on a 4 or 6% polyacrylamide/8 m urea/TBE gel for the Rpm1r and tRNA analysis, respectively. For the mRNA and rRNA analysis total RNA was separated on a 1% formaldehyde agarose gel. Blotting, hybridization, and probing for $tRNA_f^{Met}$ and Rpm1r were performed as described previously (Stribinskis *et al.* 1996). Oligonucleotide probes (Table 2) were radiolabeled with [γ-^{32}P]ATP as described (Sambrook *et al.* 1989). The signals were detected using phosphorimager scanning (PhosphorImager SF, Molecular Dynamics, Eugene, OR) or autoradiography. To quantify RNAs, the signals were normalized to that for U3 RNA.

Total yeast DNA was isolated as described (Philippsen *et al.* 1991). DNA was digested with *Hinc*II and *Taq*I, which released 1300 bp of *RPM1* and 624 bp of *ACT1*. The products were separated on an agarose gel and Southern analysis was performed (Sambrook *et al.* 1989). The *RPM1* riboprobe (above) was used to determine the amount of mitochondrial DNA; an *ACT1* oligonucleotide (Table 2) was used to determine the amount of nuclear DNA. The signals were detected using phosphorimager scanning.

Labeling of mitochondrial translation products: *In vivo* pulse labeling of mitochondrial translation products with [^{35}S]methionine (New England Nuclear, Boston) and mitochondrial isolation was essentially as described (Fox *et al.* 1991), except that log-phase cells were grown in galactose media. Cells (25–50 OD_{600}) were used for the pulse labeling with 0.1 mCi of [^{35}S]methionine, and 250–500 OD_{600} for pulse-chase labeling with 0.5 mCi [^{35}S]methionine. Labeling was stopped by adding an excess of cold methionine. The radiolabeled proteins were fractionated by SDS-PAGE in a 16.5% gel, and analysis of the dried gel was performed using phosphorimager scanning.

Extraction and spectra of mitochondrial cytochromes: Mito-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
YMW9	*MAT***a**/α *ade2-1 ade3Δ22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100*	Lutz *et al.* (2000)
YML34	*MAT***a**/α *ade2-1 ade3Δ22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 RPM2/Δrpm2::KanMX*	Lutz *et al.* (2000)
YML34.1	*MAT***a** *ade2-1 ade3Δ22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 RPM2/Δrpm2::KanMX* YEp352/*RPM2*	Lutz *et al.* (2000)
YML34.2	*MAT*α *ade2-1 ade3Δ22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 RPM2/Δrpm2::KanMX* YEp352/*RPM2*	Lutz *et al.* (2000)
YMS2	*MAT***a** *ade2-1 ade3Δ22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 Δrpm2::KanMX rpm2-100*	This study
KKY3	*MAT***a** *his3-Δ200 isp42::HIS3 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* (pRS316/*ISP42*)	Kassenbrock *et al.* (1995)
KKY3.3	*MAT***a** *his3-Δ200 isp42::HIS3 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* (pRS316/*isp42-3*)	Kassenbrock *et al.* (1995)

chondria were isolated after conversion of cells to spheroplasts (Dieckmann and Tzagoloff 1983). The mitochondria were suspended in 50 mm Tris-HCl, pH 8.0, at a protein concentration of 10 mg/ml. Cytochromes were extracted and difference spectrum was obtained as described (Tzagoloff 1995).

Mitochondrial enzyme assays: The cytochrome c oxidase and the NADH-cytochrome c reductase activities were determined spectrophotometrically as described (Coruzzi *et al.* 1979). Reduced cytochrome c was prepared by adding small amounts of sodium dithionite to a solution of horse heart cytochrome c (Sigma, St. Louis) followed by aeration to remove excess dithionite. The nonenzymatic rates were measured at 550 nm for 1 min after which 20 μg of mitochondria was added and the reaction was followed for an additional 2 min. The enzymatic rate was calculated from the difference in absorbance at 550 nm between 15 and 45 sec after addition of mitochondria. The specific activity is expressed as micromoles of cytochrome c oxidized or reduced per minute per milligram of mitochondrial protein at 23°.

Western analysis: Proteins were separated by 16.5% SDS-PAGE for the analysis of cytochrome c oxidase subunits and by 7.5% SDS-PAGE for the analysis of Rpm2 proteins using the buffer system of Laemmli, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and treated with antibodies. The monoclonal antibodies against Cox1p, Cox2p, and Cox3p were used as recommended (Molecular Probes, Eugene, OR); antibodies that recognize nuclear-encoded subunits of cytochrome c oxidase (Glerum *et al.* 1995) and antibodies against Mdh1p and Cytb (Chacinska *et al.* 2000) were also used. The anti-Rpm2p antibodies were made against a peptide encoding amino acids 306–323 (QCB Inc., Hopkinton, MA) and were used at 1:200 dilution.

Protein concentrations were determined using a Bio-Rad D_C protein assay kit (Bio-Rad, Hercules, CA).

TABLE 2

Oligonucleotides used in this study

Primer	Sequence (5′–3′)
COX1	CTCTCTTTCATAATC
COX2	GATACTAAACCTAAAATAACTAAT
COX3	CGAATTAATATCTCTTTCGCCGG
CYTb	AATTTTTATATTATTTATTAATATTGTT
ATP9	TGCTAATACTAATTGCAT
15S RNA	ATGACTCGTATGCGTCATGTCC
21S RNA	GCAACATCAACCTGTTCGATCG
U3	GGATTGCGGACCAAGCTAA
$tRNA^{Phe}$	TTTATCGCTTTACCAC
$tRNA^{Glu}$	CCATTAGACGATAAGGTC
ACT1	AGGACAAAACGGCTTGGATGGAAACG TAGAAGGCTGGAACG

RESULTS

The *rpm2-100* mutant is respiratory deficient but maintains wild-type mtDNA and grows on fermentable carbon sources: The *RPM2* mutant allele, *rpm2-100*, has a deletion in the coding region of the *RPM2* open reading frame downstream of the mitochondrial presequence such that the protein product does not contain amino acids 146–246. We introduced plasmids containing *RPM2*, *rpm2-100*, or vector alone into cells harboring a complete deletion of the *RPM2* gene on a chromosome, but carrying a wild-type *RPM2* gene on a *URA3*-containing plasmid, and then measured the ability of these cells to grow on media containing 5-fluoroorotic acid (5-FOA). Only cells that lose the *URA3*-containing plasmid and have another source of functional Rpm2p can grow under these conditions. Both *RPM2* and *rpm2-100* cells grew on plates containing 5-FOA (Figure 1A). The mutant cells grew at rates comparable to wild type on rich glucose medium (YPD; Figure 1B). Together, these results show that the mutant allele *rpm2-100* supports the essential function of *RPM2* and that amino acids 146–246 are dispensable for growth on glucose. In contrast, *rpm2-100* cells do not form visible colonies after 4 days on plates containing the nonfermentable carbon sources glycerol/ethanol (YPGE; Figure 1B). The respiratory growth deficiency of the *rpm2-100* mutant strain is leaky and colonies are observed upon prolonged incubation.

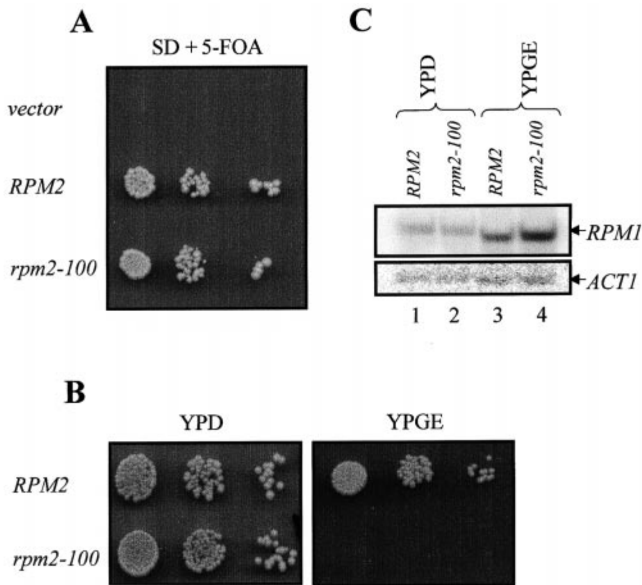

Figure 1.—Growth phenotypes and level of mtDNA in *RPM2* and *rpm2-100* cells. (A) *rpm2-100* complements the essential function of *RPM2.* Plasmids coding for Rpm2p, rpm2-100p, or vector alone were introduced into cells with *RPM2* on a *URA3*-containing plasmid. Transformants were spotted in 1:10 serial dilutions on 5-FOA plates. (B) Cells harboring the *rpm2-100* allele do not form visible colonies on nonfermentable carbon sources. Serial dilutions of *RPM2* and *rpm2-100* cells were plated and incubated for 2 days on YPD and 4 days on YPGE. (C) *rpm2-100* cells retain mtDNA. Total cellular DNA was isolated from cells grown in glucose medium (YPD) and after shifting to glycerol/ethanol medium (YPGE) for 14–16 hr. A DNA blot was sequentially probed with *RPM1* riboprobe, specific for mtDNA, and *ACT1* oligonucleotide, specific for nuclear DNA. Wild-type DNA was loaded in lanes 1 and 3, and *rpm2-100* DNA in lanes 2 and 4.

Since Rpm2p is required for the maintenance of the wild-type mitochondrial genome, defects in the integrity of mtDNA could explain the respiratory deficiency of *rpm2-100* cells. To determine whether *rpm2-100* cells maintain wild-type mtDNA, they were crossed to a wild-type strain devoid of mtDNA. The resulting diploids grow on nonfermentable carbon sources (data not shown), indicating that the *rpm2-100* mutation is recessive and that *rpm2-100* cells retain wild-type mtDNA. The amount of mtDNA relative to nuclear DNA was also examined in *rpm2-100* cells, in which the chromosomal copy of *RPM2* was replaced by *rpm2-100,* and wild-type cells. Cells were grown in glucose medium and shifted for 14–16 hr to glycerol/ethanol medium. A Southern blot with *RPM1* as a probe for mtDNA and *ACT1* as a probe specific for nuclear DNA revealed that *RPM1* gene content was comparable between wild-type (lanes 1 and 3 in Figure 1C) and mutant cells (lanes 2 and 4 in Figure 1C). Both strains appeared to increase their mtDNA copy number to the same extent when grown under derepressing conditions. Although *rpm2-100* cells maintain wild-type amounts of mtDNA, they respire poorly.

RNase P-related functions appear normal in *rpm2-100* cells: We examined Rpm1r biosynthesis and RNase P activity to determine whether alterations in the known functions of Rpm2p could account for the respiratory defect in *rpm2-100* cells. Total RNA was isolated from wild-type and *rpm2-100* cells that were grown in glucose before a shift to glycerol/ethanol medium for 14–16 hr. Northern analysis was performed with probes specific for mitochondrial $tRNA^{Met}_{f}$, $tRNA^{Phe}$, $tRNA^{Glu}$, and Rpm1r. These tRNAs represent three different mitochondrial transcription units and have either short ($tRNA^{Met}_{f}$, $tRNA^{Phe}$) or long ($tRNA^{Glu}$) 5′ leader sequences. The *rpm2-100* cells make mature mitochondrial $tRNA^{Met}_{f}$, $tRNA^{Phe}$, and $tRNA^{Glu}$ (Figure 2, A and B). Levels of mature tRNAs are comparable to wild type under both fermentative and respiratory growth conditions. There is no evidence that tRNA precursors accumulate in either strain.

Synthesis of the RNA subunit of mitochondrial RNase P, Rpm1r, is also dependent on Rpm2p (Stribinskis *et al.* 1996). To determine if the *rpm2-100* mutation affects Rpm1r synthesis, the RNA was blotted with a probe to Rpm1r (Figure 2C). Mature Rpm1r is made under all growth conditions, although it does appear that more precursors accumulate in *rpm2-100* cells. Steady-state levels of mitochondrial RNA increase after cells are shifted to nonrepressing carbon sources such as glycerol/ethanol media (Ulery *et al.* 1994). It is clear from the data in Figure 2 that this response is not defective in the *rpm2-100* cells. Since neither the amount nor the processing of mitochondrial tRNAs we examined were impaired, we decided to examine mitochondrial protein synthesis. It was possible that processing of a mitochondrial tRNA that we did not test might be defective in *rpm2-100* cells and this could lead to a reduction of mitochondrial protein synthesis. We reasoned that if translation were reduced to the point that it no longer supported respiration, but not to the point where mtDNA stability was compromised, a respiratory phenotype such as seen in *rpm2-100* cells could be observed.

Analysis of *rpm2-100* mitochondrial translation products revealed defects in the synthesis of mitochondrially encoded Cox1p, Cox2p, and Cox3p: We compared the synthesis of mitochondrial gene products in *RPM2* and *rpm2-100* strains by pulse labeling for 30 min with [^{35}S]methionine. These experiments were carried out in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Mitochondria were then isolated and proteins were analyzed by SDS-PAGE and phosphorimaging. Both strains incorporated radiolabeled methionine into mitochondrial translation products and the total amount of label incorporated was comparable (data not shown). However, while *rpm2-100* cells synthesized all major mitochondrial gene products, the relative amount of label incorporated into each of the mitochondrially encoded proteins differed between *rpm2-100* and wild-type cells (Figure 3A, lanes 1 and 2). Labeling of Cox1p, Cox2p, and Cox3p was reduced in

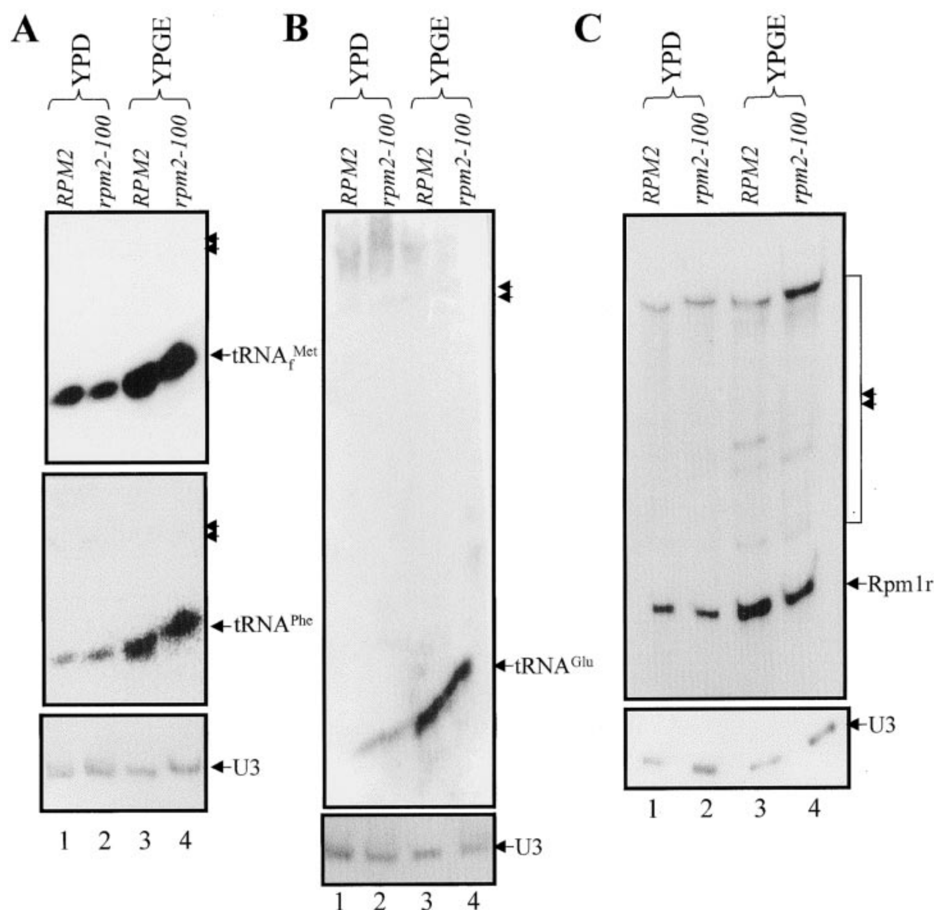

FIGURE 2.—Processing of mitochondrial tRNAs and Rpm1r. Total cellular RNA was isolated from cells grown in glucose medium (YPD) and after shifting to glycerol/ethanol medium (YPGE) for 14–16 hr. Northern blots were performed with probes to $tRNA_f^{Met}$ and $tRNA^{Phe}$ (A, top and middle, respectively), $tRNA^{Glu}$ (B), and Rpm1r (C). Nuclear-encoded U3 was used as a loading control. Wild-type RNA was loaded in lanes 1 and 3 and *rpm2-100* RNA in lanes 2 and 4. Arrowheads indicate positions of RNA precursors.

the mutant relative to wild type. The incorporation of label into Var1p in the mutant was about twofold less than that in wild type. In contrast, incorporation of label into apocytochrome b and ATPase subunit 6 was elevated in the mutant relative to wild type. Thus, *rpm2-100* cells have an altered pattern of mitochondrial protein synthesis. To determine if the labeling profiles were a consequence of differences in protein stability, cells were pulse labeled and chased for up to 6 hr in the presence of excess nonradioactive methionine. In this experiment, mitochondrial proteins were fractionated using a long gel, which allowed the separation of all major mitochondrial proteins. Figure 3B shows that the stability of radiolabeled mitochondrial proteins appeared to be comparable in both mutant (even lanes) and wild type (odd lanes). The incorporation of $[^{35}S]$methionine into all three mitochondrially encoded subunits of ATPase was more efficient in the mutant relative to wild-type cells.

To address the possibility that a pulse-labeling period shorter than 30 min might be necessary to observe equivalent rates of protein synthesis in the two strains, pulse labeling for 5 min was performed. As shown in Figure 3C, decreased amounts of Cox1, Cox2, Cox3, and Var1 proteins were observed in *rpm2-100* (lane 2) relative to wild type (lane 1). This confirms that protein synthesis, but not protein stability, is affected in *rpm2-100* cells.

The Var1 protein, a component of the yeast mitochondrial small ribosomal subunit (GROOT *et al.* 1979; TERPSTA and BUTOW 1979), was reduced in *rpm2-100* cells. However, this reduction is not limiting for the synthesis of other mitochondrially encoded proteins in *rpm2-100* mutant, since Atp6p, Atp8p, Atp9p, and Cytbp were synthesized even more efficiently compared to wild type. The observed pattern of protein synthesis indicates that a reduction in cytochrome c oxidase levels is the most likely cause of the respiratory defect in *rpm2-100* cells.

***rpm2-100* cells lack visible spectra of aa_3 cytochromes and have low cytochrome c oxidase activity:** We performed spectral analysis of cytochromes, enzyme activity assays, and immunoblot analysis using mitochondria isolated from *rpm2-100* and wild-type cells to determine the effects of the *rpm2-100* mutation on cytochrome c oxidase activity and subunit accumulation. The cytochrome composition of mutant mitochondria was determined from the visible spectrum of extracts obtained under conditions known to quantitatively solubilize all the respiratory components of the organelle (TZAGOLOFF 1995). Room temperature cytochrome spectra revealed that the absorption band corresponding to cytochromes aa_3, which reflects the amount of cytochrome c oxidase, was dramatically reduced in the mutant. In contrast, the level and absorption maxima for cyto-

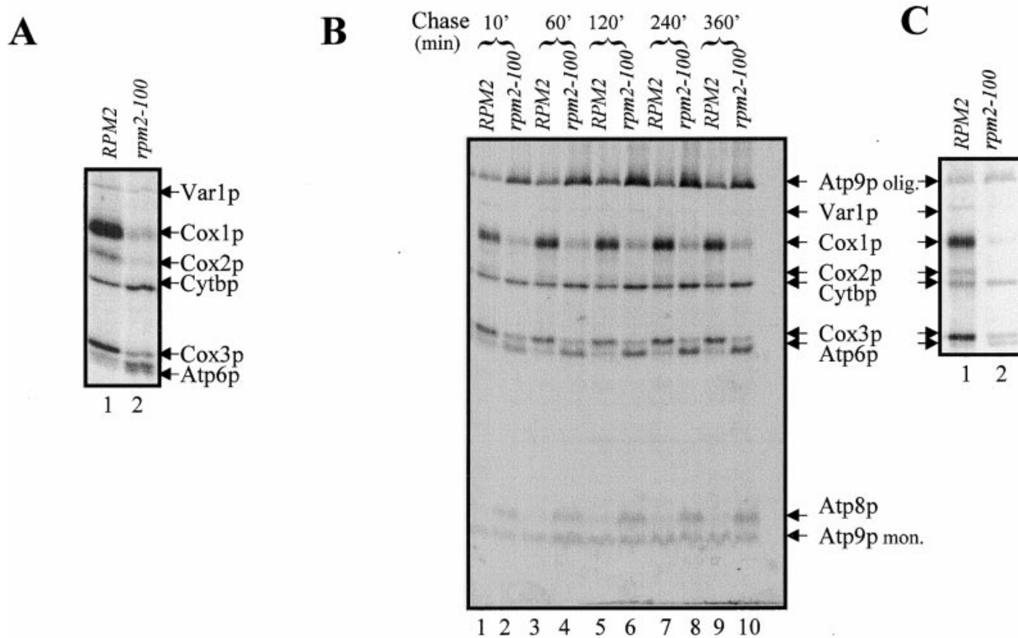

FIGURE 3.—*In vivo* synthesis of mitochondrially encoded proteins in *RPM2* and *rpm2-100* cells. Mitochondrial translation products were labeled with [^{35}S]methionine in the presence of cycloheximide for 30 min (A), or 5 min (C). Mitochondrial proteins (80 μg/lane) were resolved by SDS-PAGE in a 16.5% gel. The gel was soaked in autoradiographic image enhancer, dried, and exposed to a phosphorimager screen. (B) Stability of newly synthesized mitochondrial proteins. Cells were labeled and analyzed as in A. However, after addition of cold methionine, cells were further incubated for the indicated time periods. The identities of translation products are shown on the right: Cox1p, Cox2p, and Cox3p correspond to subunits 1, 2, and 3 of cytochrome c oxidase, respectively: Cytbp refers to the product of the cytochrome b gene; Atp6p, Atp8p, and Atp9p correspond to subunits 6, 8, and 9 of the F_0F_1-ATPase, respectively.

chromes c + c_1 and b were unaffected in *rpm2-100* cells (Figure 4A). To determine the biochemical consequence of the cytochromes aa_3 defect, we measured cytochrome c oxidase and NADH-cytochrome c reductase activities in *rpm2-100* mitochondria. Table 3 shows that *rpm2-100* cells have only 10% of the wild-type cytochrome c oxidase activity, while their NADH-cytochrome c reductase activity is normal.

Western blot analysis of the mutant cell extracts revealed low steady-state levels of the Cox1, -2, and -3 proteins, but wild-type levels of mitochondrially encoded Cytb (Figure 4B). Immunoblots also revealed that

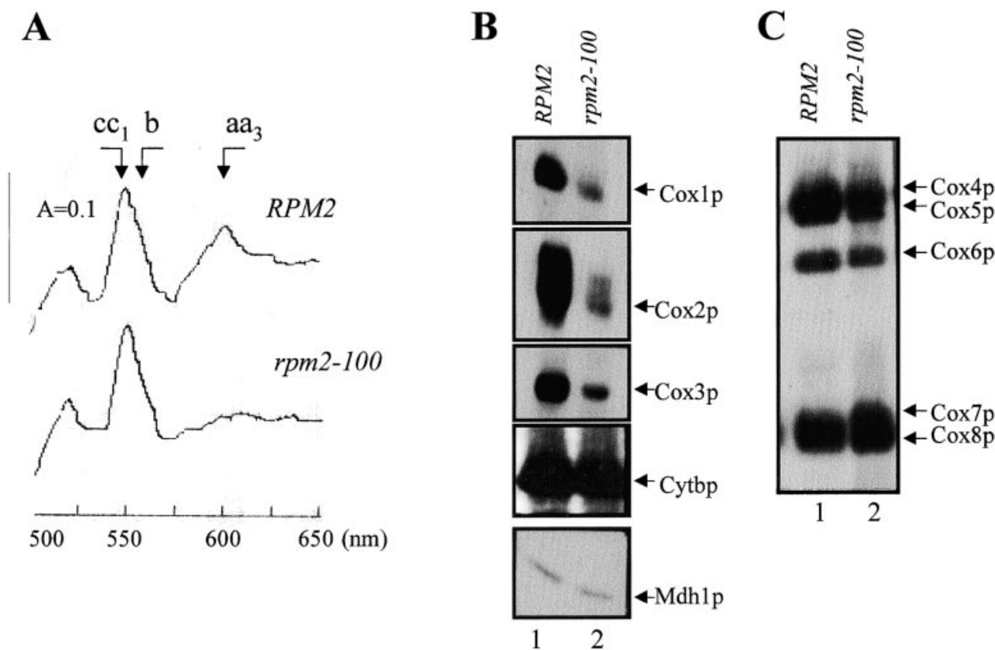

FIGURE 4.—Spectra of mitochondrial cytochromes and protein levels of cytochrome c oxidase in wild type and *rpm2-100* mutant. (A) The cytochrome composition of wild-type and mutant mitochondria was determined from the difference spectra of reduced *vs.* oxidized mitochondrial extracts recorded at room temperature. The α-absorption bands of cytochromes aa_3 have maxima at 603 nm. The corresponding maximum of cytochrome b is 560 nm and cytochrome c is 550 nm. (B) Western blot analysis of mitochondrial extracts with antibodies to the mitochondrially encoded cytochrome c oxidase subunits 1, 2, 3, and Cytp. Nuclear-encoded mitochondrial matrix protein Mdh1p was used as a loading control. (C) Western blot analysis of mitochondrial extracts with antibodies that recognize the nuclear-encoded Cox4, Cox5, Cox6, Cox7, and Cox8 proteins.

TABLE 3

Activities of cytochrome c oxidase and NADH-cytochrome c reductase

RPM2 allele	Cytochrome c oxidase[a]	NADH-cytochrome c reductase[a]
RPM2	0.55	0.92
rpm2-100	0.055	0.92

[a] The cytochrome c oxidase and NADH-cytochrome c reductase activities were determined by measuring the change in the absorbance at 550 nm with reduced or oxidized cytochrome c, respectively, after addition of 20 μg of mitochondrial protein at room temperature. The specific activity is expressed as micromoles of cytochrome c oxidized or reduced per minute per milligram mitochondrial protein.

The values indicated are averages of triplicate measurements.

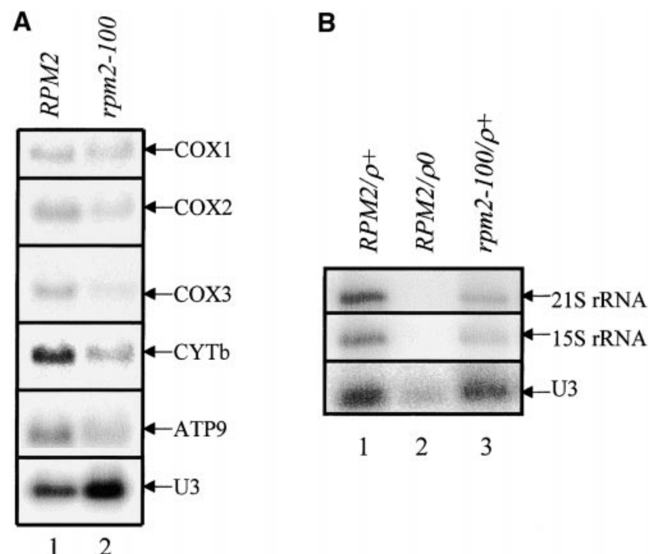

Figure 5.—Steady-state levels of mitochondrial mRNA and rRNA. Total cellular RNA was isolated from *RPM2* (lane 1) and *rpm2-100* (lane 2 in A and lane 3 in B) cells after shifting to glycerol/ethanol medium (YPGE) for 14–16 hr. A Northern blot with probes to COX1, COX2, COX3, CYTb, and ATP9 (A) and 21S rRNA and 15S rRNA (B) was performed. Total RNA isolated from cells lacking mitochondrial DNA was included in B (lane 2) as a negative control. The figure shows a computer-generated image of the phosphorimager scan. RNA levels were normalized to the levels of U3 RNA. The amounts of mature transcripts in the *rpm2-100* strain relative to wild type were as follows: COX1, 38%; COX2, 15%; COX3, 13%; CYTb, 25%; ATP9, 15%; 21S rRNA, 25%; 15S rRNA, 25%.

the levels of nuclear-encoded subunits Cox4p, Cox5p, Cox6p, Cox7p, and Cox8p in *rpm2-100* cells were comparable to wild type (Figure 4C). Thus, the respiratory deficient growth phenotype of *rpm2-100* appears to be caused by decreased levels of Cox1p, Cox2p, and Cox3p.

Steady-state levels of mRNA and rRNA were reduced in *rpm2-100* mitochondria: Differential regulation of yeast mitochondrial genes appears to take place via gene-specific controls of RNA processing, stability, and translation (Attardi and Schatz 1988; Costanzo and Fox 1990; Dieckmann and Staples 1994). To determine whether the decrease in mitochondrially encoded cytochrome c oxidase subunits resulted from a decrease in the steady-state levels of their mRNAs, total RNA was isolated from *RPM2* and *rpm2-100* cells, fractionated, blotted to a membrane, and hybridized with oligonucleotide probes specific for COX1, COX2, COX3, CYTb, ATP9 mRNA, as well as 15S and 21S rRNA (Table 2). Steady-state levels of all mature forms of mitochondrial mRNAs and rRNAs analyzed were reduced in *rpm2-100* cells relative to wild type (Figure 5). The reduction varied from 2- to 8-fold for different RNAs and did not correlate with the presence or absence of introns. The ratio of precursor to mature RNA, when precursors were observed, was comparable to those in wild-type cells. Finally, there was no direct correlation between abundance of mRNA and the synthesis of the corresponding protein during protein labeling *in vivo*. For example, despite a 4-fold reduction in mRNA for CYTb and 8-fold for ATP9, the relative incorporation of [^{35}S]methionine into apocytochrome b (Cytbp) and ATPase subunit 9 (Atp9p) in *rpm2-100* cells was greater than that in wild type. These results indicate that some proteins can be synthesized efficiently in *rpm2-100* mitochondria, even when the amount of their corresponding mRNA is reduced. Others have observed substantial decreases in mRNA levels without concomitant effects on the synthesis of protein products. For example, despite 2- to 5-fold reduction in CYTb and 20-fold reduction in COX1 mRNA levels in *SUV3-1*, the synthesis of Cox1 and Cytb polypeptides was about the same as that in wild-type cells (Conrad-Webb *et al.* 1990). Two other reports demonstrate that COX2 mRNA levels are not limiting for Cox2p synthesis, and 40-fold reduction still allowed growth by respiration (Pinkham *et al.* 1994; Dunstan *et al.* 1997). Finally, Cliften *et al.* (1997) showed that mitochondrial transcription is reduced at higher temperatures and concluded that mitochondrial function can be maintained with only 10–20% of wild-type transcript levels. Therefore, the 2- to 8-fold reduction of mRNAs and the 4-fold reduction of rRNAs should not preclude appreciable growth on nonfermentable carbon sources. These considerations suggest that the major effect of the *rpm2-100* mutation is at the translational level.

***RPM2*, but not *rpm2-100*, is a high-copy suppressor of *tom40-3*:** *RPM2* was isolated as a high-copy suppressor of *tom40-3*, a temperature-sensitive allele coding for a component of the mitochondrial protein import channel (Kassenbrock *et al.* 1995). It was clear in these previous experiments that RNase P activity was not required for high-copy suppression of *tom40-3*. However, all alleles that provided the essential function also suppressed the *tom40-3* allele. Although the mechanism of

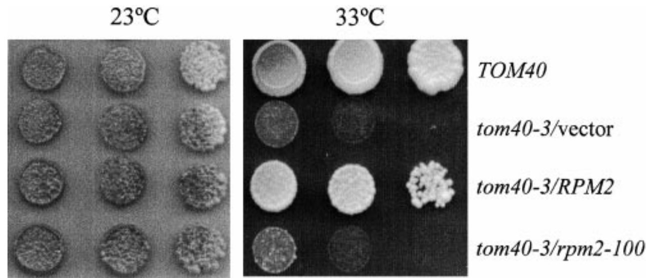

Figure 6.—The *rpm2-100* mutation abolishes the ability of Rpm2p to suppress the defect in *tom40-3* cells. Multi-copy plasmids coding for Rpm2p, rpm2-100p, or vector alone, were introduced into cells carrying the *tom40-3* temperature-sensitive allele. Transformants were spotted in 1:10 serial dilutions on YPD plates. Plates were incubated at 23° and 33° (permissive and nonpermissive temperature, respectively, for the *tom40-3* allele) for 5 days.

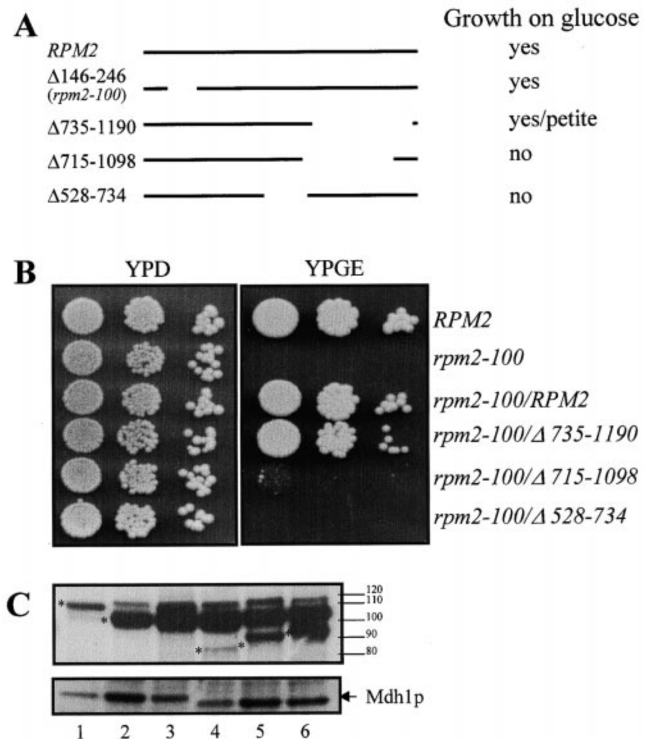

Figure 7.—Full-length Rpm2p is not required for suppression of *rpm2-100*. (A) A schematic representation of *RPM2* alleles. The deleted amino acid residues are indicated on the left. Low-copy plasmids containing the deletion alleles were transformed into *YML34.1* (*rpm2::KAN*, YEp352/*RPM2*) and streaked onto 5-FOA plates to assess the growth phenotypes in the absence of wild-type *RPM2* (right column). Yes, indicates growth comparable to wild type; yes/petite, indicates complementation, but cells lose wild-type mtDNA at high frequency; no, indicates lack of complementation. (B) Expression of the amino-terminal portion of Rpm2p suppresses the *rpm2-100* mutant phenotype. The *rpm2-100* cells transformed with different *RPM2* alleles on low-copy plasmid were plated in 1:10 serial dilutions on YPD and YPGE and scored for growth. (C) Mutant Rpm2p proteins were expressed and targeted to mitochondria. Mitochondrial proteins were isolated from all six strains shown in B and probed for Rpm2p. The nuclear-encoded mitochondrial protein Mdh1p was used as a loading control. Lane 1, *RPM2*; lane 2, *rpm2-100*; lane 3, *rpm2-100/RPM2*; lane 4, *rpm2-100/Δ735-1190*; lane 5, *rpm2-100/Δ715-1098*; lane 6, *rpm2-100/Δ528-735*. Asterisks indicate the migration of Rpm2p proteins. The upper band in lanes 2 and 4–6 is most likely a precursor form of rpm2-100 protein. Protein molecular mass standards are indicated on the right.

suppression of *tom40-3* is, as yet, unclear, it does suggest a model relating Rpm2p function to mitochondrial import, which is an essential feature of mitochondrial biogenesis. To determine whether *rpm2-100* could also serve as a high-copy suppressor of *tom40-3*, we transformed the mutant with *rpm2-100* on a high-copy vector. Figure 6 shows that *rpm2-100* is not a high-copy suppressor of *tom40-3*.

***RPM2* alleles that complement the *rpm2-100* respiratory defect also provide the function essential for growth on glucose:** The fermentative growth function of Rpm2p has been localized to the amino-terminal 734 amino acids of the *RPM2* reading frame. The same portion of Rpm2p also suppressed the *tom40-3* temperature-sensitive growth when provided on a high-copy vector (Kassenbrock *et al.* 1995). To determine if the novel function reported here can be uncoupled from the previously identified unknown function necessary for growth on all carbon sources, we asked whether mutant alleles of *RPM2* that support the essential function also complement the respiratory growth defect of *rpm2-100* cells. Deletions were introduced to remove increasing amounts of the carboxy terminus (Δ735-1190 and Δ715-1098) or 206 amino acids in the middle (Δ528-734) of Rpm2p. The ability of these alleles to support growth on the fermentable carbon source glucose was tested using plasmid shuffling (Figure 7A). *rpm2.Δ735-1190* has the same phenotype as an insertional disruption of chromosomal *RPM2* at the *Hpa*I site (*rpm2::LEU2*; Morales *et al.* 1992). Cells lose mtDNA at high frequency, but grow well on glucose. *rpm2.Δ715-1098* and *rpm2.Δ528-734* cells have phenotypes comparable to Δ*rpm2* and are unable to grow on glucose medium (Figure 7A). Each allele was transformed into yeast containing chromosomal *rpm2-100* as its only source of Rpm2p. Figure 7B shows that wild-type *RPM2* and *rpm2.Δ735-1190*, but not *rpm2.Δ715-1098* or *rpm2.Δ528-734*, could complement the *rpm2-100* respiratory growth defect. We used the same strains to determine whether stability or mitochondrial localization of Rpm2p was affected by the different mutations. Western analysis (Figure 7C) showed that alleles that do not provide function do produce detectable amounts of the mutant proteins that are localized to mitochondria. In fact, these alleles generally produced higher amounts of Rpm2 protein than the functional derivative rpm2.Δ735-1190p or wild-type Rpm2p. Therefore, because only alleles of *RPM2* that provide the function essential for fermentative growth complement *rpm2-100*, we conclude that these two functions are related.

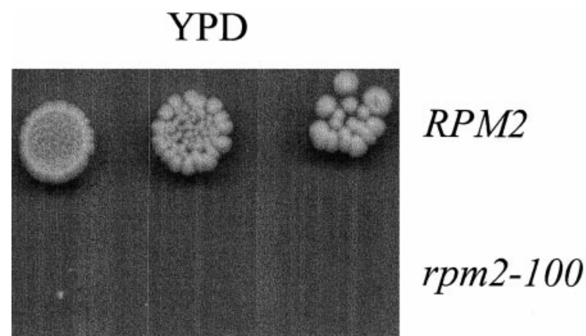

FIGURE 8.—The *rpm2-100* cells do not form visible colonies on YPD plates after treatment with ethidium bromide. *RPM2* and *rpm2-100* strains were grown in YPD liquid medium in the presence of 20 μg/ml of EB for 48 hr and were plated in 1:10 serial dilutions on YPD and incubated for 4 days.

The *rpm2-100* mutation converts *S. cerevisiae* to a petite negative yeast: Mitochondrial RNase P is not essential for growth on fermentable carbon sources in the yeast *S. cerevisiae*. Petite mutants completely lacking mtDNA are capable of growth on fermentable carbon sources. To determine the consequences of mtDNA depletion in the *rpm2-100* background, we cultivated wild-type and *rpm2-100* cells in glucose medium in the presence of ethidium bromide (EB). EB is a potent mitochondrial mutagen that causes deletions in mtDNA (Slonimski *et al.* 1968). After 48 hr of cultivation with EB, aliquots of cells were plated on solid glucose medium without EB, and their ability to form colonies was determined. Figure 8 shows that *rpm2-100* cells do not form visible colonies on YPD plates after 4 days, while *RPM2* cells do. Only after prolonged incubation (over a week) did some cells give rise to colonies. Cells from these colonies grew at variable (slow) rates on glucose and were unable to grow on nonfermentable carbon sources (data not shown). This phenotype is reminiscent of Δ*rpm2* mutants, which display a significant rate of phenotypic reversion on glucose-containing medium (Kassenbrock *et al.* 1995; Lutz *et al.* 2000). Thus, *rpm2-100* cells undergo a limited number of cell divisions and arrest growth after they lose mtDNA. This result indicates that the fermentative growth of *rpm2-100* cells is dependent on the maintenance of the wild-type mitochondrial genome. Therefore, we conclude that the RNase P activity retained in *rpm2-100* cells becomes essential for growth on glucose, through its effect on the maintenance of the wild-type mitochondrial genome.

DISCUSSION

Rpm2p is a multifunctional protein required for mitochondrial RNase P activity and for fermentative growth. Cells lacking mitochondrial RNase P activity lose wild-type mtDNA and thereby respiratory competence but grow by fermentation (Morales *et al.* 1992). This dual requirement has prevented a detailed characterization of the role of *RPM2* in mitochondrial biogenesis. We describe here an allele of *RPM2*, *rpm2-100*, that supports mitochondrial RNase P activity and yet is unable to support normal respiratory growth. Cells harboring the *rpm2-100* allele are deficient in the synthesis of mitochondrially encoded cytochrome c oxidase subunits Cox1p, Cox2p, and Cox3p. The resultant decrease in cytochrome c oxidase activity provides an explanation for the respiratory deficiency observed in *rpm2-100* mutant cells. Thus, *RPM2* has at least two separable functions that contribute to respiratory growth.

The defect in mitochondrial protein synthesis in *rpm2-100* cells is selective. Cox1p, Cox2p, and to a lesser extent Cox3p and Var1p are decreased in the mutant relative to wild type, whereas Cytbp and ATPase subunits 6, 8, and 9 are increased. There are a number of nuclear genes that affect the synthesis of specific mitochondrial gene products (Attardi and Schatz 1988; Tzagoloff and Dieckmann 1990; Dieckmann and Staples 1994; Pel and Grivell 1994; Fox 1996). Most, if not all, of the eight major mitochondrially encoded mRNAs are translated under the direction of mRNA-specific translational activator proteins specified by nuclear genes (Fox, 1996). Each mitochondrial mRNA appears to require its own specific activators for translation. The best studied, COX3 mRNA, is specifically activated by a complex containing the nuclear encoded gene products Pet54p, Pet122p, and Pet494p, (Müller *et al.* 1984; Costanzo and Fox 1986; Costanzo *et al.* 1986; Fox *et al.* 1988; Brown *et al.* 1994). The Pet111p nuclear gene product (Poutre and Fox 1987; Mulero and Fox 1993) specifically activates translation of COX2 mRNA, whereas mutations in two genes, *MSS51* (Decoster *et al.* 1990) and *PET309* (Manthey and McEwen 1995) block translation of COX1 mRNA. Interestingly, Rpm2p has sequence similarity with Pet309p over a region spanning residues 189–302 of Rpm2p. This region, which contains 49% sequence similarity with Pet309p, partially overlaps with residues 146–246 deleted in *rpm2-100*. Thus, it is possible that in addition to its role as a component of mitochondrial RNase P, Rpm2p could act as a translational activator for the mitochondrially encoded cytochrome c oxidase genes.

RPM2 joins a number of nuclear genes involved in mitochondrial RNA processing that play a second role in mitochondrial biogenesis or function. *NAM2* encodes the leucyl-tRNA synthetase and *NAM2* is also required for the splicing of the COB bI4 and COX1 aI4 introns (Herbert *et al.* 1988). The splicing defect in *PET54* mutants can be suppressed in intron-less strains, even though the remaining defect in the translation of COX3 does not allow respiratory competence (Valencik *et al.* 1989), indicating a dual mitochondrial function for *PET54*. The genetic evidence suggests that different functional domains of the Pet54 protein facilitate expression of the mitochondrial genes *COX1* and *COX3* (Valencik and McEwen 1991). The product of the

MSS18 gene is required for the splicing of the COX1 aI5β intron by promoting the cleavage of the 5′ exon-intron junction (Seraphin *et al.* 1988). However, strains missing the aI5β intron and *MSS18* grow only half as well on lactate as those with *MSS18*. This suggests that *MSS18* has a second, as yet unidentified, respiratory function (Seraphin *et al.* 1988). The nuclear gene *MSS116* has also been implicated in the splicing of several introns of both COX1 and CYTb, as well as having an additional role in the translation of mitochondrial encoded genes (Seraphin *et al.* 1989). For each of these genes, as well as for *RPM2*, the dual functions of their proteins can be at least partially separated by genetics. Some of these genes have a direct role in translation, while others, including *RPM2*, may act indirectly.

A role of Rpm2p in translation of mitochondrial COX mRNAs does not, however, readily explain the role of Rpm2p in fermentative growth, since growth on glucose is not dependent on the mitochondrial *COX* genes or their translational activators. Therefore, it is likely that the defect in the synthesis of mitochondrially encoded proteins is a reflection of some other process that, when disrupted, leads to mitochondrial dysfunction. In this context, a defect in the synthesis of mitochondrially encoded cytochrome c oxidase subunits was reported for mutants of the nuclear gene *SSC1*, which encodes mitochondrial Hsp70, a protein that plays an important role in the folding and assembly of proteins that are either newly imported or synthesized within the organelle (Kang *et al.* 1990; Manning-Krieg *et al.* 1991; Herrmann *et al.* 1994; Westermann *et al.* 1996). *SSC1*, like several other components of the mitochondrial import apparatus, is an essential gene. When Hsp70 function is altered, the pattern of proteins synthesized in mitochondria changes. While mitochondrial translation continues in *ssc1-2* and *ssc1-3* mutants at the nonpermissive temperature, the amount of Cox1, -2, and -3 proteins is reduced relative to wild-type cells (Herrmann *et al.* 1994).

A link between Rpm2p and the mitochondrial import apparatus was established when *RPM2* was isolated as a high-copy suppressor of *tom40-3*, which encodes a temperature-sensitive component of the mitochondrial protein import channel (Kassenbrock *et al.* 1995). Tom40 is an essential protein of the outer membrane translocase (Baker *et al.* 1990) and forms the basic import core (Hill *et al.* 1998; van Wilpe *et al.* 1999). Several studies have shown that proteins destined for import into mitochondria can be unfolded once engaged by the import apparatus. This unfolding has been assigned to the outer membrane translocation apparatus (Mayer *et al.* 1995). Mitochondrial preproteins are in close contact with Tom40p and associate with the translocation machinery by interaction through both the presequence and the mature portion of the protein (Rapaport *et al.* 1997, 1998). This intimate contact maintains the preprotein in a translocation-competent form. It has also been shown that unfolding is associated with the protease-resistant part of the translocation machinery rather than with the surface receptors, which characterize the protein import complex as a membrane-integrated chaperone (Mayer *et al.* 1995). The observation that multiple copies of *RPM2* can suppress a mutation in a key component of this complex, while *rpm2-100* cannot, suggests that the same function of Rpm2p required for the synthesis of mitochondrially encoded subunits of cytochrome c oxidase within the organelle might be required for *tom40-3* suppression. If the defect in cytochrome c oxidase synthesis in *rpm2-100* cells is related to a chaperone function of Rpm2p within the organelle, this same activity could underlie the mechanism of suppression of the *TOM40* mutant at the mitochondrial surface. Alternatively, Rpm2p may be involved in the synthesis of nuclear-encoded chaperones involved in mitochondrial biogenesis and function. A critical feature of either model infers an extramitochondrial localization of a fraction of Rpm2p.

The function of *RPM2* compromised by the *rpm2-100* mutation makes yeast cells dependent on the wild-type mitochondrial genome for fermentative growth. A small number of proteins required for respiratory growth have been shown to be essential for growth on fermentable carbon sources in the absence of wild-type mtDNA. These genes include *AAC2* (Kováč *et al.* 1967), *PGS1* (Subik 1974), *YME1* (Weber *et al.* 1995), and genes encoding α- and β-subunits of F_1-ATPase (Chen and Clark-Walker 1999a). Little is known regarding the mechanism linking the maintenance of an intact mitochondrial genome to the function of these genes, since a common primary defect is unknown (for review see Chen and Clark-Walker 1999b; Contamine and Picard 2000).

Nonetheless, each of these genes can be tied in, either directly or indirectly, to the mitochondrial import process. *AAC2* encodes the major ADP/ATP carrier and defects in its activity could alter adenine nucleotide pools and thereby affect import, since the import of proteins into mitochondria needs energy available inside the organelle (Nelson and Schatz 1979). *PGS1* catalyzes the first step in a biosynthetic pathway of phosphatidylglycerol and cardiolipin (Chang *et al.* 1998), two anionic phospholipids that are confined mainly to mitochondrial membranes (Zinser *et al.* 1991). Anionic phospholipids participate in the formation of α-helices in the presequences of mitochondrial proteins (Wang and Weiner 1994; Chupin *et al.* 1996) and appear to facilitate the unfolding of proteins during import into mitochondria (Eilers *et al.* 1989; Endo *et al.* 1989). The α-subunit of F_1-ATPase (encoded by *ATP1*) shares sequence similarity with molecular chaperones (Luis *et al.* 1990; Alconada *et al.* 1994) and is required for the normal function of the inner membrane in promoting efficient protein import (Yuan and Douglas 1992). Finally, there is a genetic interaction between *ATP1*

and *YME1*, which suggests that there could be some functional overlap between F_1-ATPase and Yme1p. *YME1* encodes a putative ATP and zinc-dependent protease localized to the mitochondrial inner membrane (Weber *et al.* 1996) and has a potential role as a molecular chaperone (Nakai *et al.* 1995). The inability of *yme1* mutants to grow in the absence of wild-type mtDNA can be suppressed by mutations in the γ-subunit of F_1-ATPase (Weber *et al.* 1995). These mutations are identical to those in the petite-negative yeast *Kluyveromyces lactis*, which convert it to petite-positive yeast (Chen and Clark-Walker 1995), and the authors have proposed that a novel property of the F_1 complex other than ATP hydrolysis is responsible for the suppression (Chen and Clark-Walker 1999a). Thus, it is possible that mutations in these genes, as well as in *RPM2*, may decrease the efficiency of mitochondrial import. If the efficiency is further reduced by the loss of the mitochondrial genome and the concurrent reduction in membrane potential, import efficiency may be reduced below a point consistent with supporting growth on both nonfermentable and fermentable carbon sources.

The link between the function lost by the *rpm2-100* mutation and the dependence on a wild-type mitochondrial genome provides an explanation for why a complete deletion of *RPM2* prevents growth on fermentable carbon sources. The mitochondrial RNase P is required for the maintenance of the wild-type mitochondrial genome. Mitochondrial genomes in cells lacking mtRNase P activity accumulate deletions or are lost completely after a limited number of divisions. Cells compromised in the second Rpm2p function characterized here require the wild-type mitochondrial genome and are unable to grow in the absence of RNase P activity. Thus, a complete deletion of *RPM2* causes lethality because mutations in the two functions of Rpm2p in combination are lethal, while either one can support growth on glucose.

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LITERATURE CITED

Alconada, A., A. I. Flores, L. Blanco and J. M. Cuezva, 1994 Antibodies against F1-ATPase alpha-subunit recognize mitochondrial chaperones: evidence for an evolutionary relationship between chaperonin and ATPase protein families. J. Biol. Chem. **269:** 13670–13679.

Attardi, G., and G. Schatz, 1988 Biogenesis of mitochondria. Annu. Rev. Cell Biol. **4:** 289–333.

Baker, K. P., and G. Schatz, 1991 Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature **349:** 205–208.

Baker, K. P., H. Schneider, B. Guiard, M. Brunner and G. Schatz, 1990 A yeast mitochondrial outer membrane protein is essential for protein import and cell viability. Nature **348:** 605–609.

Brown, N. G., M. C. Costanzo and T. D. Fox, 1994 Interactions among three proteins that specifically activate translation of mitochondrial *COX3* mRNA in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **14:** 1045–1053.

Chacinska, A., M. Boguta, J. Krzewska and S. Rospert, 2000 Prion-dependent switching between respiratory competence and deficiency in the yeast *nam9*-1 mutant. Mol. Cell. Biol. **20:** 7220–7229.

Chang, S.-C., P. N. Heacok, C. J. Clancey and W. Dowhan, 1998 The *PEL1* gene (renamed *PGS1*) encodes the phosphatidylglycerophosphate synthase of *Saccharomyces cerevisiae.* J. Biol. Chem. **273:** 9829–9836.

Chen, D. C., B. C. Yang and T. T. Kuo, 1992 One step transformation of yeast in stationary phase. Curr. Genet. **21:** 83–84.

Chen, X. J., and G. D. Clark-Walker, 1995 Specific mutations in α and γ-subunits of F_1-ATPase affect mitochondrial genome integrity in the petite-negative yeast *Kluyveromyces lactis.* EMBO J. **14:** 3277–3286.

Chen, X. J., and G. D. Clark-Walker, 1999a α and β subunits of F_1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae.* Mol. Gen. Genet. **262:** 898–908.

Chen, X. J., and G. D. Clark-Walker, 1999b The petite mutation in yeast: 50 years on. Int. Rev. Cytol. **194:** 197–238.

Chupin, V., J. M. Leenhouts, A. I. De Kroon and B. De Krujff, 1996 Secondary structure and topology of a mitochondrial presequence peptide associated with negatively charged micelles. A 2D[1] H-NMR study. Biochemistry **35:** 3141–3146.

Cliften, P. F., J.-Y. Park, B. P. Davis, S.-H. Jang and J. A. Jaehning, 1997 Identification of three regions essential for interaction between a σ-like factor and core RNA polymerase. Genes Dev. **11:** 2897–2909.

Conrad-Webb, H., P. S. Perlman, H. Zhu and R. A. Butow, 1990 The nuclear *SUV3-1* mutation affects a variety of post transcriptional processes in yeast mitochondria. Nucleic Acids Res. **18:** 1369–1376.

Contamine, V., and M. Picard, 2000 Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol. Mol. Biol. Rev. **64:** 281–313.

Coruzzi, G., M. K. Trembath and A. Tzagoloff, 1979 The isolation of mitochondrial and nuclear mutants of *Saccharomyces cerevisiae* with specific defects in mitochondrial functions. Methods Enzymol. **56:** 95–106.

Costanzo, M. C., and T. D. Fox, 1986 Product of *Saccharomyces cerevisiae* nuclear gene *PET494* activates translation of a specific mitochondrial mRNA. Mol. Cell. Biol. **6:** 3694–3703.

Costanzo, M. C., and T. D. Fox, 1990 Control of mitochondrial gene expression in *Saccharomyces cerevisiae.* Annu. Rev. Genet. **24:** 91–113.

Costanzo, M. C., E. C. Seaver and T. D. Fox, 1986 At least two nuclear gene products are specifically required for translation of a single yeast mitochondrial mRNA. EMBO J. **5:** 3637–3641.

Dang, Y. L., and N. C. Martin, 1993 Yeast mitochondrial RNase P. Sequence of the *RPM2* gene and demonstration that its product is a protein subunit of the enzyme. J. Biol. Chem. **268:** 19791–19796.

Decoster, M., M. Simon, D. Hatat and G. Faye, 1990 The *MSS51* gene product is required for the translation of the *COX1* mRNA in yeast mitochondria. Mol. Gen. Genet. **224:** 111–118.

Dieckmann, C. L., and A. Tzagoloff, 1983 Analysis of yeast mitochondrial genes. Methods Enzymol. **97:** 361–373.

Dieckmann, C. L., and R. R. Staples, 1994 Regulation of mitochondrial gene expression in *Saccharomyces cerevisiae.* Int. Rev. Cytol. **152:** 145–181.

Dunstan, H. M., N. S. Green-Willms and T. D. Fox, 1997 *In vivo* analysis of *Saccharomyces cerevisiae* COX2 mRNA 5′-untranslated leader functions in mitochondrial translation initiation and translational activation. Genetics **147:** 87–100.

Eilers, M., T. Endo and G. Schatz, 1989 Adriamycin, a drug interacting with acidic phospholipids, blocks import of precursor proteins by isolated yeast mitochondria. J. Biol. Chem. **264:** 2945–2950.

Endo, T., M. Eilers and G. Schatz, 1989 Binding of the tightly folded artificial mitochondrial precursor protein to the mitochondrial outer membrane involves a lipid-mediated conformational change. J. Biol. Chem. **264:** 2951–2956.

Fox, T. D., 1996 Genetics of mitochondrial translation, pp. 733–758 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fox, T. D., M. C. Costanzo, C. A. Strick, D. L. Marykwas, E. C. Seaver *et al.*, 1988 Translational regulation of mitochondrial gene expression by nuclear genes of *Saccharomyces cerevisiae.* Philos. Trans. R. Soc. Lond. B **319:** 97–105.

Fox, T. D., L. S. Foley, J. J. Mulero, T. W. Thorsness, P. E. Hedin *et al.*, 1991 Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol. **194:** 149–165.

Glerum, D. M., T. J. Koerner and A. Tzagoloff, 1995 Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase. J. Biol. Chem. **270:** 15585–15590.

Grivell, L. A., M. Artal-Sanz, G. Hakkaart, L. De Jong, L. G. J. Nijtmans *et al.*, 1999 Mitochondrial assembly in yeast. FEBS Lett. **452:** 57–60.

Groot, G. S. P., T. L. Mason and N. V. Harten-Loosbroek, 1979 Var1 is associated with the small ribosomal subunit of mitochondrial ribosomes in yeast. Mol. Gen. Genet. **174:** 339–342.

Herbert, C., M. Labouesse, G. Dujardin and P. Slonimski, 1988 The *NAM2* proteins from *S. cerevisiae* and *S. douglasii* are mitochondrial leucyl-tRNA synthetases and are involved in mRNA slicing. EMBO J. **7:** 473–483.

Herrmann, J. M., and W. Neupert, 2000 Protein transport into mitochondria. Curr. Opin. Microbiol. **3:** 210–214.

Herrmann, J. M., R. A. Stuart, E. A. Craig and W. Neupert, 1994 Mitochondrial heat shock protein 70, a molecular chaperone for proteins encoded by mitochondrial DNA. J. Cell Biol. **127:** 893–902.

Hill, K., K. Model, M. T. Ryan, K. Dietmeier, F. Martin *et al.*, 1998 Tom40 forms the hydrophilic channel of the mitochondrial import pore preproteins. Nature **395:** 516–521.

Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig *et al.*, 1990 Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature **348:** 137–143.

Kassenbrock, C. K., G.-J. Gao, K. R. Groom, P. Sulo, M. G. Douglas *et al.*, 1995 *RPM2*, independently of its mitochondrial RNase P function, suppresses an *ISP42* mutant defective in mitochondrial import and is essential for normal growth. Mol. Cell. Biol. **15:** 4763–4770.

Kohrer, K., and H. Domdey, 1991 Preparation of high molecular weight RNA. Methods Enzymol. **194:** 398–405.

Kováč, L., T. M. Lachowicz and P. Slonimski, 1967 Biochemical genetics of oxidative phosphorylation. Science **158:** 1564–1567.

Luis, A. M., A. Alconada and J. M. Cuezva, 1990 The alpha regulatory subunit of the mitochondrial F_1-ATPase complex is a heat-shock protein: identification of two highly conserved amino acid sequences among the alpha-subunits and molecular chaperones. J. Biol. Chem. **265:** 7713–7716.

Lutz, M. S., S. R. Ellis and N. C. Martin, 2000 Proteosome mutants, *pre4-2* and*ump1-2*, suppress the essential function but not the mitochondrial RNase P function of the *Saccharomyces cerevisiae* gene *RPM2.* Genetics **154:** 1013–1023.

Manning-Krieg, U. C., P. E. Scherer and G. Schatz, 1991 Sequential action of mitochondrial chaperones in protein import into the matrix. EMBO J. **10:** 3273–3280.

Manthey, G. M., and J. E. McEwen, 1995 The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae.* EMBO J. **14:** 4031–4043.

Mayer, A., W. Neupert and R. Lill, 1995 Mitochondrial protein import: reversible binding of the presequence at the *trans* side of the outer membrane drives partial translocation and unfolding. Cell **80:** 127–137.

Miller, D. L., and N. C. Martin,1983 Characterization of the yeast mitochondrial locus necessary for tRNA biosynthesis: DNA sequence analysis and identification of a new transcript. Cell **34:** 911–917.

Morales, M. J., Y. L. Dang, Y. C. Lou, P. Sulo and N. C. Martin, 1992 A 105-kDa protein is required for yeast mitochondrial RNase P activity. Proc. Natl. Acad. Sci. USA **89:** 9875–9879.

Mulero, J. J., and T. D. Fox, 1993 *PET111* acts in the 5′-leader of the *Saccharomyces cerevisiae COX2* mRNA to promote its translation. Genetics **133:** 509–516.

Müller, P. P., M. K. Reif, S. Zonghou, C. Sengstag, T. L. Mason *et al.*, 1984 A nuclear mutation that post-transcriptionally blocks accumulation of a yeast mitochondrial gene product can be suppressed by a mitochondrial gene rearrangement. J. Mol. Biol. **175:** 431–452.

Myers, A. M., L. K. Pape and A. Tzagoloff, 1985 Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae.* EMBO J. **4:** 2087–2095.

Nakai, T., T. Yasuhara, Y. Fujuki and A. Ohashi, 1995 Multiple genes, including a member of the AAA family, are essential for degradation of unassembled subunit 2 of cytochrome c oxidase in yeast mitochondria. Mol. Cell. Biol. **15:** 4441–4452.

Nelson, N., and G. Schatz, 1979 Energy-dependent processing of cytoplasmically made precursors to mitochondrial proteins. Proc. Natl. Acad. Sci. USA **76:** 4365–4369.

Pel, H. J., and L. A. Grivell, 1994 Protein synthesis in mitochondria. Mol. Biol. Rep. **19:** 183–194.

Philippsen, P., A. Stotz and C. Scherf, 1991 DNA of *Saccharomyces cerevisiae.* Methods Enzymol. **194:** 169–182.

Pinkham, J. L., A. M. Dudley and T. L. Mason, 1994 T7 RNA polymerase-dependent expression of COXII in yeast mitochondria. Mol. Cell. Biol. **14:** 4643–4652.

Pon, L., and G. Schatz, 1991 Biogenesis of yeast mitochondria, pp. 333–406 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics*, edited by J. R. Broach, J. R. Pringle and E. W. Jones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Poutre, C. G., and T. D. Fox, 1987 *PET111*, a *Saccharomyces cerevisiae* nuclear gene required for translation of the mitochondrial mRNA encoding cytochrome c oxidase subunit II. Genetics **115:** 537–547.

Rapaport, D., W. Neupert and R. Lill, 1997 Mitochondrial protein import. Tom40 plays a major role in targeting and translocation of preproteins by forming a specific site for the presequence. J. Biol. Chem. **272:** 18725–18731.

Rapaport, D., A. Mayer, W. Neupert and R. Lill, 1998 *cis* and *trans* sites of the TOM complex of mitochondria in unfolding and initial translocation of preproteins. J. Biol. Chem. **273:** 8806–8813.

Rothstein, R., 1991 Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:** 281–301.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seraphin, B., M. Simon and G. Faye, 1988 *MSS18*, a yeast nuclear gene involved in the splicing of intron aI5β of the mitochondrial *cox1* transcript. EMBO J. **7:** 1455–1464.

Seraphin, B., M. Simon, A. Boulet and G. Faye, 1989 Mitochondrial splicing requires a protein from a novel helicase family. Nature **337:** 84–87.

Sherman, F., G. R. Fink and J. B. Hicks, 1986 *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and J. D. Boeke, 1991 *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. Methods Enzymol. **194:** 302–318.

Slonimski, P. P., G. Perrodin and J. H. Croft, 1968 Ethidium bromide induced mutation of yeast mitochondria: Complete transformation of cells into respiratory deficient nonchromosomal "petites". Biochem. Biophys. Res. Commun. **30:** 232–239.

Stribinskis, V., G.-J. Gao, P. Sulo, Y. L. Dang and N. C. Martin, 1996 Yeast mitochondrial RNase P RNA synthesis is altered in an RNase P protein subunit mutant: insights into the biogenesis of a mitochondrial RNA-processing enzyme. Mol. Cell. Biol. **16:** 3429–3436.

Subik, J., 1974 A nuclear mutant of *S. cerevisiae* non-tolerating the cytoplasmic petite mutation. FEBS Lett. **42:** 309–312.

Terpsta, P., and R. A. Butow, 1979 The role of Var1 in the assembly of yeast mitochondrial ribosomes. J. Biol. Chem. **254:** 12662–12669.

Tzagoloff, A., 1995 Ubiquinol-cytochrome-c oxidoreductase from *Saccharomyces cerevisiae.* Methods Enzymol. **260:** 51–63.

Tzagoloff, A., and C. L. Dieckmann, 1990 *PET* genes of *Saccharomyces cerevisiae.* Microbiol. Rev. **54:** 211–225.

Ulery, T. L., S. H. Jang and J. A. Jaehning, 1994 Glucose repression of yeast mitochondrial transcription: kinetics of derepression and role of nuclear genes. Mol. Cell. Biol. **14:** 1160–1170.

Underbrink-Lyon, K., D. L. Miller, N. A. Ross, H. Fukuhara and N. C. Martin, 1983 Characterization of a yeast mitochondrial locus necessary for tRNA biosynthesis. Mol. Gen. Genet. **191:** 512–518.

Valencik, M. L., and J. E. McEwen, 1991 Genetic evidence that different functional domains of the *PET54* gene product facilitate expression of the mitochondrial genes *COX1* and *COX3* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 2399–2405.

Valencik, M. L., B. Kloeckener-Gruissem, R. O. Poyton and J. E. McEwen, 1989 Disruption of the yeast nuclear *PET54* gene blocks excision of mitochondrial intron aI5β from pre-mRNA for cytochrome c oxidase subunit I. EMBO J. **8:** 3899–3904.

van Wilpe, S., M. Ryan, K. Hill, A. C. Maarse, C. Meisenger *et al.*, 1999 Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase. Nature **401:** 485–489.

Wang, Y., and H. Weiner, 1994 Evaluation of electrostatic and hydrophobic effects on the interaction of mitochondrial signal sequences with phospholipid bilayers. Biochemistry **33:** 12860–12867.

Weber, E. R., R. S. Rooks, K. S. Shafer, J. W. Chase and P. E. Thorsness, 1995 Mutations in the mitochondrial ATP synthase gamma subunit suppress a slow-growth phenotype of yme1 yeast lacking mitochondrial DNA. Genetics **140:** 435–442.

Weber, E. R., T. Hanekamp and P. E. Thorsness, 1996 Biochemical and functional analysis of the *YME1* gene product, an ATP and zinc-dependent mitochondrial protease from *S. cerevisiae*. Mol. Biol. Cell **7:** 307–317.

Westermann, B., B. Gaume, J. M. Herrmann, W. Neupert and E. Schwarz, 1996 Role of the mitochondrial DnaJ homologue Mdj1p as a chaperone for mitochondrially synthesized and imported proteins. Mol. Cell. Biol. **16:** 7063–7071.

Yuan, H., and M. G. Douglas, 1992 The mitochondrial F_1-ATPase alpha-subunit is necessary for efficient import of mitochondrial precursors. J. Biol. Chem. **267:** 14696–14702.

Zinser, E., C. D. Sperka-Gottlieb, E. V. Fasch, S. D. Kohlwein, F. Paltauf *et al.*, 1991 Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J. Bacteriol. **173:** 2026–2034.

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