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Rpm2p, a Component of Yeast Mitochondrial RNase P, Acts as a Transcriptional Activator in the Nucleus

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Rpm2p, a protein subunit of yeast mitochondrial RNase P, has another function that is essential in cells lacking the wild-type mitochondrial genome. This function does not require the mitochondrial leader sequence and appears to affect transcription of nuclear genes. Rpm2p expressed as a fusion protein with green fluorescent protein localizes to the nucleus and activates transcription from promoters containing lexA-binding sites when fused to a heterologous DNA binding domain, lexA. The transcriptional activation region of Rpm2p contains two leucine zippers that are required for transcriptional activation and are conserved in the distantly related yeast *Candida glabrata*. The presence of a mitochondrial leader sequence does not prevent a portion of Rpm2p from locating to the nucleus, and several observations suggest that the nuclear location and transcriptional activation ability of Rpm2p are physiologically significant. The ability of *RPM2* alleles to suppress *tom40-3*, a temperature-sensitive mutant of a component of the mitochondrial import apparatus, correlates with their ability to transactivate the reporter genes with lexA-binding sites. In cells lacking mitochondrial DNA, Rpm2p influences the levels of *TOM40*, *TOM6*, *TOM20*, *TOM22*, and *TOM37* mRNAs, which encode components of the mitochondrial import apparatus, but not that of *TOM70* mRNA. It also affects *HSP60* and *HSP10* mRNAs that encode essential mitochondrial chaperones. Rpm2p also increases the level of Tom40p, as well as Hsp60p, but not Atp2p, suggesting that some, but not all, nucleus-encoded mitochondrial components are affected.

In the yeast *Saccharomyces cerevisiae*, mitochondrial RNase P, a 5′ tRNA-processing enzyme, is coded in both mitochondrial and nuclear genomes. Mitochondrial DNA (mtDNA) codes for Rpm1r, the RNA subunit of mitochondrial RNase P, and nuclear DNA codes for the protein subunit, Rpm2p (18, 27, 45, 67). Moreover, Rpm2p is also required for maturation of the RNA subunit, Rpm1r (62), and separate domains of Rpm2p promote tRNA and Rpm1r maturation (63). Analysis of a mutant allele, *rpm2-100*, revealed that *RPM2* has another mitochondrial function, in addition to the mitochondrial RNase P-related functions (64). Cells with *rpm2-100* as their only source of Rpm2p have correctly processed mitochondrial tRNAs but are still respiration deficient. 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 (64). Thus, Rpm2p has another role in mitochondrial biogenesis, in addition to its role as a subunit of mitochondrial RNase P.

Surprisingly, there is a synthetic lethal interaction between *rpm2-100* and the loss of wild-type mtDNA (64). Cells with either the *rpm2-100* mutation or the deletion of mtDNA grow on glucose. When both alterations occur in the same cell, there is no growth on any carbon source. This explains why a complete deletion of *RPM2* is lethal (32). Loss of RNase P activity leads to loss of mtDNA, and loss of the second function requires maintenance of mtDNA for cell viability. Therefore, the *RPM2* essential function is conditional and depends on the status of the mitochondrial genome.

A link between a non-RNase P function of Rpm2p and mitochondrial protein import was established when *RPM2* was isolated as a high-copy-number suppressor of *tom40-2*, which encodes a temperature-sensitive component of the mitochondrial import channel (2, 32). The RNase P activity of Rpm2p is not required for suppression (32). This result is substantiated by the observation that *rpm2-100* supports normal RNase P activity but does not suppress *tom40-3* (64). Therefore, this non-RNase P function of Rpm2p could stem from a second role for Rpm2p within the mitochondria, or it could be the result of a function for Rpm2p elsewhere in the cell.

Here we present data that a portion of Rpm2p is nuclear. We also demonstrate that Rpm2p has a transcriptional activation domain and plays a role in defining the steady-state levels of mRNAs for some nucleus-encoded mitochondrial components. Furthermore, the ability of Rpm2p to act as a transcriptional activator in the nucleus strongly correlates with its ability to support a non-RNase P function of *RPM2*.

MATERIALS AND METHODS

Strains, media, and reagents. Standard yeast manipulations were used (30). Yeast cells were transformed with plasmid DNA using a lithium acetate method (12). The plasmid shuffle protocol was performed as previously described (60). Rich medium included 1% Bacto Yeast Extract, 2% Bacto Peptone, and 2% glucose (YPD) or 3% glycerol and 2% ethanol instead of glucose. Synthetic complete (SD) medium lacking appropriate amino acids for plasmid retention

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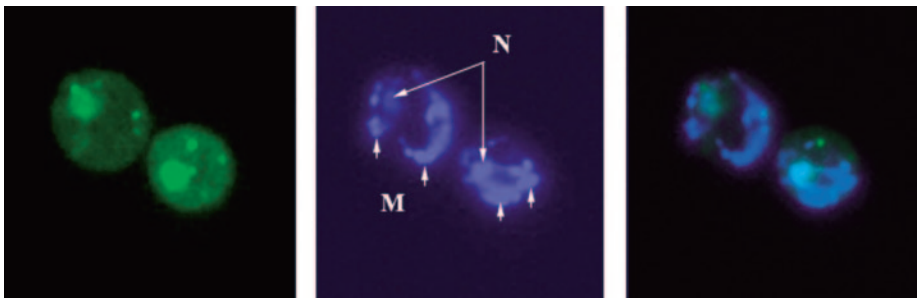

FIG. 1. Microscopy. Yeast carrying *GFP-RPM2* on a high-copy-number plasmid (left panel), stained with DAPI (middle panel), and merged (right panel). N, nuclei; M, mitochondria.

contained 0.67% Bacto Nitrogen Base and 2% glucose. Solid medium for plates included 2% Bacto Agar. Synthetic complete medium containing 1 g/liter of 5-fluoroorotic acid (5-FOA) was used to select Ura$^-$ yeast segregants. Culture medium reagents were supplied by Fisher Scientific (Pittsburgh, PA) or Difco (Detroit, MI). The yeast strains used in this study were YML34.1 (*MAT***a** *ade2-1 ade3*Δ*22 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 RPM2*/Δ*rpm2::KanMX YEp352*/*RPM2*) (41), KKY3.3 [*MAT***a** *his3-*Δ*200 isp42::HIS3 leu2-3,112 ade2-101 suc2-*Δ*9 trp1-*Δ*901 ura3-52*(pRS316/*isp42-3*)] (32), L40 [*MAT***a** *ade2 his3*Δ*200 leu2-3,112 trp1-901 LYS2::*(*lexAop*)$_4$-*HIS3 URA3::*(*lexAop*)$_8$-*lacZ gal4 gal80*] (26), BY4741 (*MAT***a** *his3*Δ *leu2*Δ *lys2*Δ *met15*Δ *ura3*Δ), and an isogenic haploid containing an *ADA3* disruption (*MAT***a** *his3*Δ *leu2*Δ *lys2*Δ *met15*Δ *ura3*Δ *ada3*Δ) generated by the *S. cerevisiae* genome deletion project consortium and were obtained from Research Genetics.

Plasmid construction. Standard procedures were used for the preparation and ligation of DNA fragments and recovery of plasmid DNA from *Escherichia coli* (55). Restriction and modification enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA; MBI Fermentas, Vilnius, Lithuania). Plasmid DNA and DNA fragments were purified using QIAGEN kits (QIAGEN, Chatsworth, CA). To construct plasmids harboring lexA-*RPM2*, lexA-*rpm2*-Δ*C*, lexA-NLS-*RPM2*, and lexA-NLS-*rpm2*-Δ*C*, original constructs for yeast two-hybrid analysis, pAS2-*RPM2* and pACT-*rpm2*-Δ*C*, were cut with XmaI and SalI. Fragments carrying *RPM2* were gel purified and ligated into pLex-a and pLexN-a (a gift from Anne Vojtek) precut with SmaI and SalI. To construct plasmids carrying portions of N-terminally truncated *RPM2* fused to lexA, PCR products were obtained with the following oligonucleotides: common (primes at ADH terminator of lexA-*rpm2*-Δ*C*), CCCCCGAGCTCATGCTATACCTGAGAAAG; 7 (amino acid 521), GCGGAATTCTTACTGCATCCAATCGGTG; 8 (amino acid 591), GCGGAATTCGCTGAGTTTATCAAGAAGAG; 9 (amino acid 644), GCGGAATTCAGCTATAATGGGCTAATATCA; 10 (amino acid 684), GCGGAATTCACTTACCCAATTTTGCAAAATG. The underlined sequences in the oligonucleotides indicate the coding sequences for either the *ADH* terminator (common) or *RPM2* (7 to 10). PCR products were digested with EcoRI and SacI and ligated into pRS314/lexA-*rpm2*-Δ*C* precut with EcoRI and SacI. To construct MLS-lexA-*RPM2*, MLS-lexA-*rpm2-100*, MLS-lexA-*rpm2*-Δ*C*, and MLS-lexA-*rpm2*-Δ*C*(−*20*), a lexA coding sequence was obtained by PCR with the following oligonucleotides: forward, TCTACCGGCCGTCCGGGCGGAATGAAAGCGTTA; reverse, TAATGTACGGCCGAATTCCAGCCAGTCGCCGTT (underlined are coding sequences). PCR products were cut with EagI and ligated into an EagI site introduced downstream of the mitochondrial leader sequence in 314/*RPM2*, 314/*rpm2-100*, 314/*rpm2*-Δ*C*, and 314/*rpm2*-Δ*C*(−*20*), respectively. To construct the *GFP-RPM2*-containing plasmid, the parental vector pPS811 was modified to eliminate the *NPL3* gene. A portion of the coding region of *RPM2* (coding for amino acids 44 to 735) was inserted into a blunted BamHI site.

Real-time PCR. Total RNA was isolated by hot-phenol extraction (34). Two micrograms of total RNA was used for cDNA synthesis with an iScript cDNA synthesis kit in a 40-μl reaction volume (Bio-Rad). One microliter of cDNA was then amplified in a total volume of 50 μl containing 1× SYBR green PCR mix (iQ SYBR green Supermix; Bio-Rad) and 300 nM gene-specific primers (sequences are available upon request). The thermal cycling conditions comprised an initial denaturation step of 95°C for 3 min and 40 cycles of 95°C for 30 s and 61°C for 45 s. All reactions were performed in triplicate. Normalization-quantification was performed using the comparative (ΔC_T) method as described in reference 48. *ATP2* was used as a reference gene because it was not affected by either *SEF1* or *RPM2*. Briefly, the C_T is the fractional cycle number for which the amount of amplified target reaches a fixed threshold. This amount is a constant depending on the primer set. The difference (ΔC_T) between the C_Ts of the target gene and the reference gene depends on the RNA relative copy number between the target and the reference gene. The amount of target normalized to an endogenous reference, *ATP2*, and relative to a calibrator ($X_{N,C}$), wild-type [*rho*$^+$] cells, is given by the equation $X_{N,C} = 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the difference between the ΔC_T of the sample and the ΔC_T of the calibrator.

Western analysis. Proteins were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the buffer system of Laemmli, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and treated with antibodies. The anti-Rpm2p antibodies were made against a peptide encoding amino acids 306 to 323 (QCB Inc., Hopkinton, MA) and were used at a 1:200 dilution. Antiserum (MD9) against Atp2p and antiserum (159-3) against Tom40p (a gift from M. G. Douglas) were used at a 1:1,000 dilution. Anti-yeast hsp60 antibodies were obtained from Stressgen Biotechnologies, Victoria, British Columbia, Canada. Protein concentrations were determined using a Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA).

Microscopy. Yeast carrying *GFP-RPM2* on a high-copy-number plasmid was grown in liquid glycerol-ethanol-containing synthetic medium lacking uracil. Expression of the *GFP-RPM2* fusion gene was induced by addition of galactose (2% final concentration), and cells were harvested, fixed, and stained with 4′,6′-diamidino-2-phenylindole (DAPI). Fluorescent images were examined using an Axiovert 200 microscope (Zeiss). The photo in Fig. 1 is of strain YMW1 grown on raffinose and switched to galactose for 45 min. The same results were obtained with BY4741.

RESULTS

A portion of Rpm2p is nuclear. Biochemical and genetic studies from our laboratory have clearly established that Rpm2p is located in mitochondria. Furthermore, Huh et al. (28) demonstrated that an Rpm2p-green fluorescent protein (GFP) fusion protein colocalizes with mitochondria, a result consistent with the known mitochondrial function of Rpm2p. However, Rpm2p has a second, essential function required in cells with dysfunctional mitochondria (64). We created a GFP-Rpm2 fusion protein, but unlike the construct of Huh et al. (28), we placed the GFP tag at the amino-terminal end by fusing the GFP coding sequence to the open reading frame 44 amino acids downstream from the initiator AUG. The Rpm2p used in this construct also lacks residues 736 to 1122, which are not required for growth by fermentation (32). Figure 1 shows the location of this fusion protein in cells grown in glycerol-ethanol-containing synthetic medium after *GFP-RPM2* gene induction with galactose. There is no mitochondrial staining because this construct lacks the mitochondrial leader sequence and has *GFP* at the amino terminus. GFP-Rpm2p produced from this construct is clearly nuclear. In addition, there are foci of GFP-Rpm2p in the cytoplasm that do not appear to colocalize with any organelle.

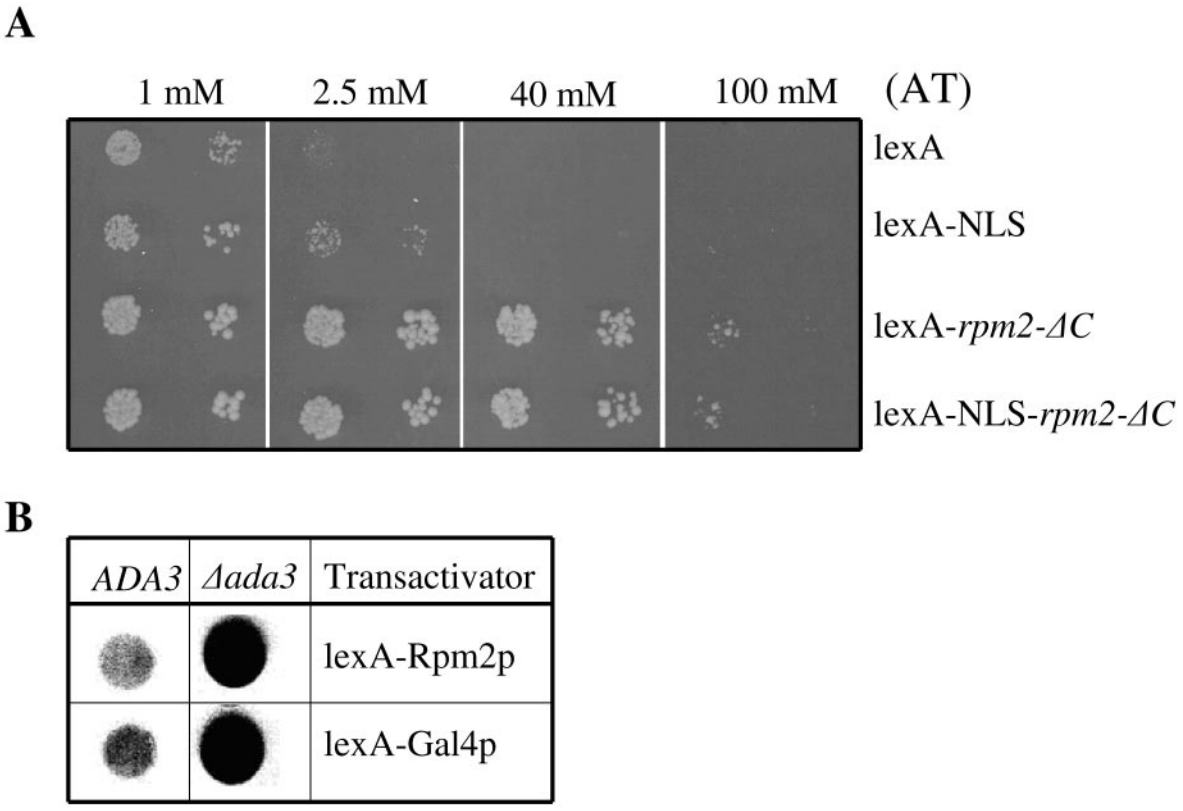

FIG. 2. Transcriptional activation by lexA-Rpm2p. (A) Complementation assay. Plasmids encoding lexA or lexA fusion proteins were introduced into the L40 yeast strain by selecting for Trp^+ colonies. Ten to 15 individual colonies were combined and grown in Trp^- liquid medium and plated on Trp^- His^- medium containing the indicated concentrations of AT, a competitive inhibitor of the *HIS3* gene product. (B) Enhanced transcriptional activation by lexA-Rpm2p in an *ADA3* deletion strain. Yeast strains expressing either lexA-Rpm2p or lexA-Gal4p activate the reporter *lacZ* on the indicator plates. Blue color intensity (appears black in the image shown) qualitatively reflects the amount of *lacZ* produced from the reporter construct.

Expression of Rpm2p as a lexA-Rpm2 fusion protein results in transcriptional activation of promoters containing lexA-binding sites. Two observations suggested that the function of Rpm2p needed for growth on glucose might be linked to a role for this protein in nuclear transcription. First, we observed high backgrounds when Rpm2p, fused to the DNA binding domain of Gal4p, was used as bait in the yeast two-hybrid screen. This suggests that Rpm2p has endogenous transcriptional activation activity. Furthermore, similar to the behavior of Gal4-VP16, Gal4-E1A, Gal4-σ^{54}, and Gal4–interleukin-1 fusion proteins, powerful transcriptional activators in yeast (9, 11, 59, 61), overexpression of the Gal4-Rpm2p fusion protein is toxic. Second, we found that multiple copies of *SEF1*, a gene encoding a putative transcription factor with a Zn(2)-Cys(2) binuclear cluster motif, suppresses *RPM2* deletions and allows cells to grow by fermentation (23).

To test the idea that Rpm2p has transcriptional activity, we created fusion proteins. We employed two reporter genes, *HIS3* and *lacZ*, that are under transcriptional control of the bacterial lexA operator. We made fusion constructs between the bacterial lexA protein and amino acids 44 to 735 of Rpm2p. In addition, we made constructs with the simian virus 40 (SV40) nuclear localization signal (NLS) inserted between the lexA and Rpm2p domains of the fusion proteins. The constructs were named lexA-NLS-*rpm2*-Δ*C* and lexA-*rpm2*-Δ*C* to reflect the presence or absence of the NLS, respectively. Like the GFP fusion protein described above, the Rpm2p used in these constructs lacks a portion of the amino-terminal mitochondrial leader sequence and residues 736 to 1202, which are not required for growth on glucose (32). A construct was made with lexA fused to the NLS alone to assay for transcriptional activation attributed to lexA in yeast. The different constructs were transformed into the reporter strain L40 (26) and transcriptional activation monitored by growth on medium lacking histidine and containing 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. Figure 2A shows that, in contrast to *Gal4-RPM2* fusions, lexA-*RPM2* fusions do not inhibit cell growth when expressed from either plasmid pLex-a or pLexN-a. Cells transformed with lexA alone grow on medium containing 1 mM AT, but growth ceases at higher concentrations of AT. This background activity is likely linked to the passive diffusion of lexA into the nucleus and a modest ability of lexA alone to activate transcription in yeast. Fusion of an NLS to lexA increased reporter activity somewhat above that seen with lexA alone, presumably due to higher nuclear accumulation of the lexA-NLS. In contrast, the lexA-*RPM2* constructs showed robust reporter activity that was only inhibited by extremely high concentrations of AT. The reporter activity observed with the lexA-*rpm2*-Δ*C* fusions was not affected by the presence or absence of the SV40 NLS. These data

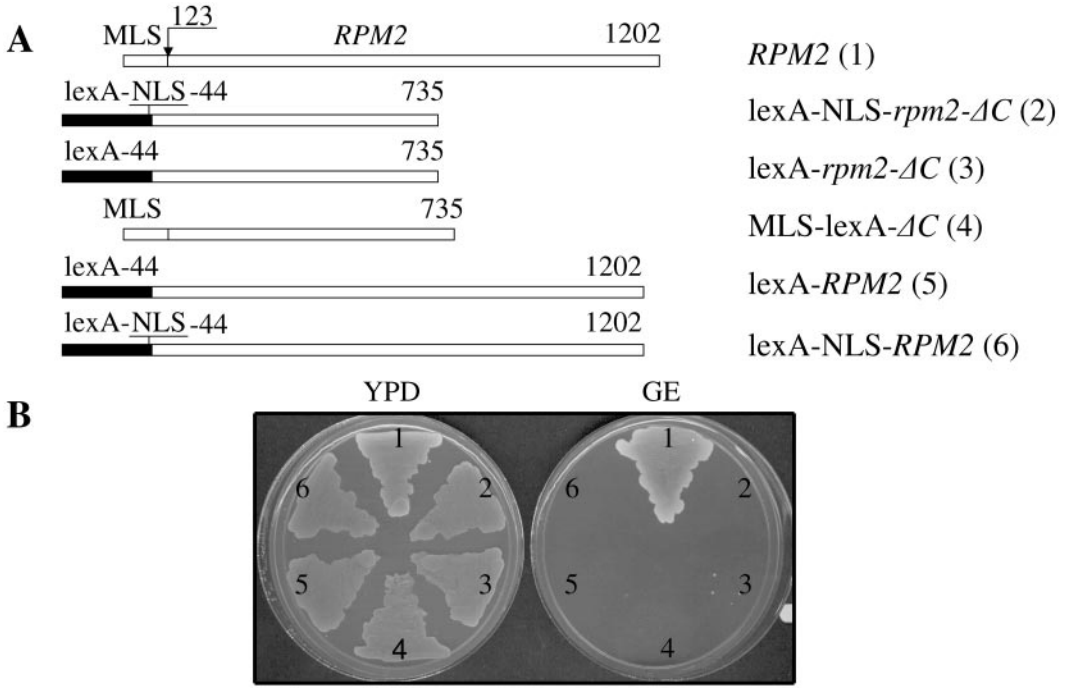

FIG. 3. Growth phenotypes of cells expressing lexA-Rpm2 fusion proteins. (A) Schematic diagram of various lex-Rpm2 fusion protein constructs. (B) Yeast expressing either wild-type *RPM2* (strain 1), lexA-NLS-*rpm2*-Δ*C* (strain 2), lexA-*rpm2*-Δ*C* (strain 3), *rpm2*-Δ*C* (strain 4), lexA-*RPM2* (strain 5), or lexA-NLS-*RPM2* (strain 6) as its only source of *RPM2* grows on glucose-containing medium (YPD). Only cells that express full-length Rpm2 protein with an intact mitochondrial leader sequence grow on nonfermentable carbon sources (glycerol and ethanol [GE]).

confirm that Rpm2p contains information needed for nuclear localization and strongly suggest it plays a role in transcriptional activation.

Although there is a well-established correlation between the degree of AT resistance and the level of *HIS3* mRNA (25), we also examined the expression of the gene for β-galactosidase, another reporter gene. In addition, to determine other requirements for transcription mediated by Rpm2p, we compared the level of transcriptional activation by lexA-Rpm2p in wild-type and *ADA3* mutant yeast. *ADA3* is a member of the histone acetyltransferase complex SAGA and is required for the integrity of SAGA (3). The Gcn5 histone acetyltransferase (8), along with SAGA/ADA complex proteins (Ada1p, Ada2p, and Ada3p), is required for some, but not all, yeast activator proteins (22, 54). Disruption of *ADA3* leads to diminished transcription by Gcn4p (50) but enhanced transcription by Gal4p, Pdr1p, and Pdr3p (6, 42). Therefore, we asked whether the SAGA complex is associated with Rpm2p transactivation ability. To address this question, we transformed wild-type and *ADA3* deletion strains with low-copy-number plasmids carrying either lexA-*rpm2*-Δ*C* or lexA-*GAL4AD*, together with the plasmid harboring *lacZ*, a reporter gene under control of the lexA operators. Transformants were grown on selective liquid medium, and the same amount of cells was spotted onto the indicator plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), a substrate of the *lacZ* gene product. In this in vivo assay, if the lexA fusion protein activates *lacZ* expression, cells will turn blue on the indicator plates. Figure 2B shows that lexA-Rpm2p does activate transcription of the *lacZ* reporter in both wild-type and *ADA3* deletion strains. The loss of *ADA3* leads to a substantial increase in expression of *lacZ* in cells producing either lexA-Rpm2 or lexA-Gal4 fusion protein. The latter has been reported previously (6). Therefore, *ADA3*, a specific transcriptional coactivator-corepressor protein represses transcriptional activation by Rpm2p.

lexA-Rpm2 fusion proteins provide the essential function of Rpm2p. We wanted to determine whether the genes producing lexA-Rpm2 proteins that were located in the nucleus and supported transcriptional activation could also substitute for *RPM2*. Since lexA-*rpm2*-Δ*C* constructs are missing a portion of the Rpm2p mitochondrial leader, we predicted that they would not be imported and the cells would be deficient in respiration. Thus, these constructs could be useful in determining whether the essential function stems from a second role for Rpm2p in mitochondria or whether it is the result of a function elsewhere in the cell, for instance, in the nucleus, as suggested by the results in Fig. 1 and 2.

In addition to the C-terminally truncated lexA-*rpm2*-Δ*C* constructs used above, we created additional lexA-*RPM2* constructs containing the full-length C terminus, with and without the heterologous SV40 NLS (Fig. 3A). We transformed cells containing a chromosomal deletion of *RPM2* and a copy of *RPM2* on a *URA3* plasmid with a *TRP1* plasmid containing either wild-type *RPM2* or *rpm2*-Δ*C*, which supports growth on glucose but not RNase P activity (63), or different lexA-*RPM2* constructs all lacking residues 1 to 44. The transformants were grown on 5-FOA to counterselect against the *URA3*-based plasmid. Only cells that lose the *URA3*-containing plasmid and

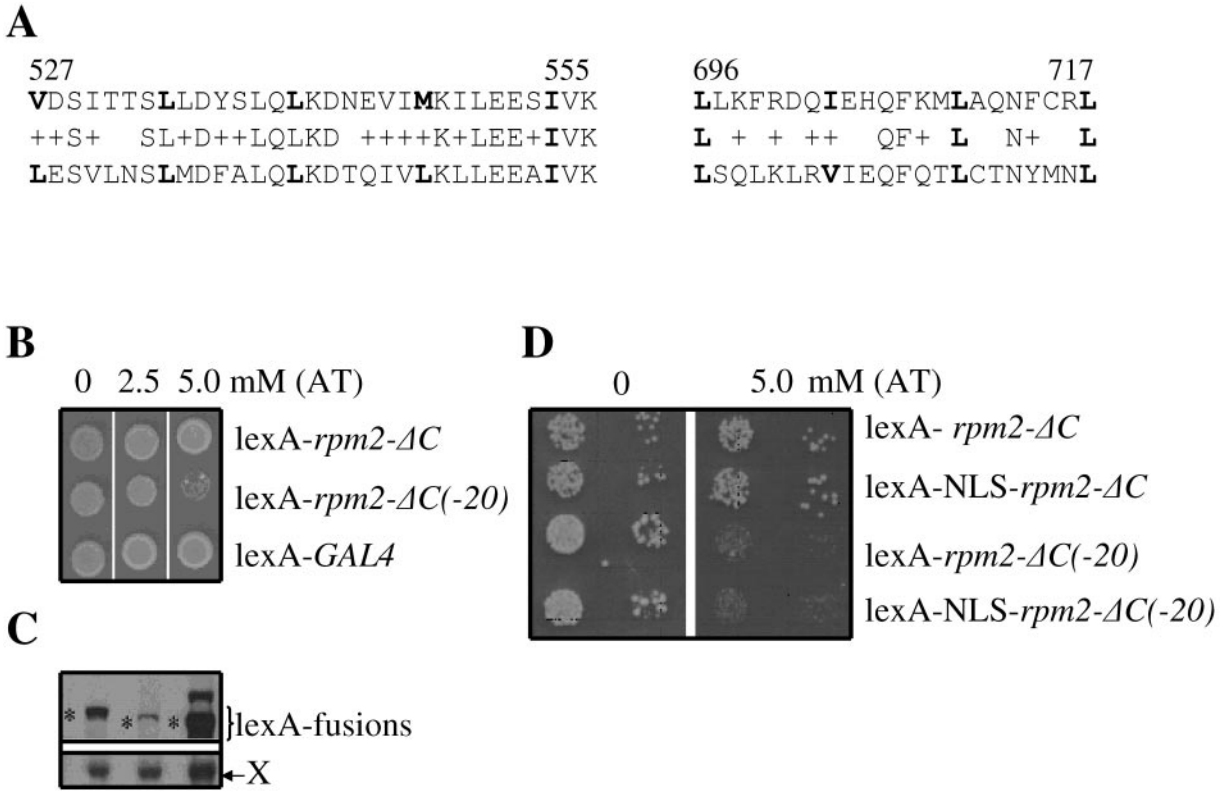

FIG. 4. Deletion of amino acids 715 to 735 diminishes the ability of Rpm2p to activate transcription as a lexA fusion protein in the L40 strain. (A) Comparison of *S. cerevisiae* and *C. glabrata* Rpm2p in the region coding for the two putative leucine zippers. In the leucine zipper, every seventh amino acid is in boldface. (B) Growth of lexA-*rpm2*-Δ*C*-, lexA-*rpm2*-Δ*C*(−*20*)-, and lexA-*GAL4*-transformed cells on medium in the absence of histidine and in the presence of AT. (C) The top part shows a Western analysis of fusion proteins detected using antibodies against lexA protein. lexA-Rpm2.ΔCp, lexA-Rpm2.ΔC(−20), and lexA-Gal4p are in lanes 1 to 3, respectively. The bottom part shows a Western analysis of unknown yeast protein, marked with the letter X, which is recognized by lexA antibodies serving as a loading control. (D) Growth of lexA-*rpm2*-Δ*C*-, lexA-NLS-*rpm2*-Δ*C*-, lexA-*rpm2*-Δ*C*(−*20*)-, and lexA-NLS-*rpm2*-Δ*C*(−*20*)-transformed cells on histidine-lacking medium without AT or with 5.0 mM AT.

have another source of functional Rpm2p can grow under these conditions. All of the strains containing either carboxyl-terminally truncated or full-length *RPM2* formed colonies on 5-FOA plates (data not shown) and continued to grow when transferred to a fresh glucose plate. Figure 3B shows that lexA-*RPM2* fusion genes coding either amino acids 44 to 735 (lexA-*rpm2*-Δ*C*) or amino acids 44 to 1202 (lexA-*RPM2*) grow comparably to those containing *RPM2* sequences alone. The presence or absence of the SV40 NLS also does not affect growth.

In contrast, cells containing lexA-*RPM2* constructs that are missing the first 1 to 43 amino acids do not grow on nonfermentable carbon sources, indicating that mitochondrial RNase P activity is lost. Like lexA-*RPM2*-containing cells, cells with *rpm2*-Δ*C* do not grow on nonfermentable carbon sources (Fig. 3B). The product of the *rpm2*-Δ*C* allele is localized to mitochondria and supports RNase P function only when maintained on nonfermentable carbon sources (63). Since mitochondrial protein synthesis is required for the maintenance of the wild-type mtDNA (44), wild-type mtDNA is lost if cells are grown first on glucose (63). Thus, the ability of lexA-*RPM2* to complement growth on fermentable carbon sources in an *rpm2* null strain indicates that expression of *RPM2*, as a lexA-Rpm2 fusion protein, does not compromise its essential function and suggests that mitochondrial localization is not required for growth on glucose.

The transcriptional activation region of Rpm2 contains two conserved leucine zipper domains, both required for transcriptional activation. Our primary structure analyses identified two putative leucine zipper domains at amino acid residues 527 to 555 and 696 to 717 in Rpm2p (Fig. 4A). Both leucine zippers are also present in a homolog from *Candida glabrata*, isolated in our laboratory by functional complementation of *S. cerevisiae RPM2* (7; GenBank accession no. AF338039) (Fig. 3A). The second leucine zipper domain is included in the lexA-*rpm2*-Δ*C* construct used above, which terminates at position 735. We have shown previously that Rpm2p truncated at amino acid 735 supports growth on glucose, whereas a mutant form 20 residues shorter, truncated at amino acid 714, no longer supports growth on glucose (63). The latter truncation includes part of the putative leucine zipper domain. To examine whether the inability of this truncation to support growth on glucose correlates with its ability to act as a transcription activator, we truncated the lexA-*RPM2* construct at residue 714 and assayed it for the ability to activate transcription from the *HIS3* reporter gene. Figure 4B shows

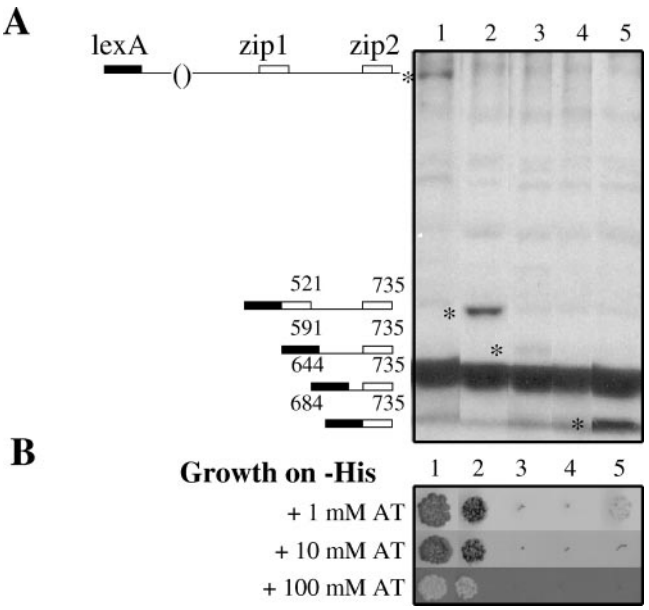

FIG. 5. (A) Schematic drawing of the deletion mutants and Western analysis showing the fusion proteins marked with asterisks. Lanes: 1, lexA-*rpm2*-Δ*C*; 2, lexA-*rpm2*$^{521-735}$; 3, lexA-*rpm2*$^{591-735}$; 4, lexA-*rpm2*$^{644-735}$; 5, lexA-*rpm2*$^{684-735}$. (B) Growth of lexA-*rpm2* deletion mutants in the absence of histidine and in the presence of increasing amounts of AT. The numbering is the same as in panel A.

that the ability of lexA-*rpm2*-Δ*C*(−*20*) to activate *HIS3* transcription is substantially diminished relative to lexA-*rpm2*-Δ*C*, but the fusion protein is made, albeit in a reduced amount (Fig. 4C). These data indicate that the ability of Rpm2p to support growth on glucose correlates with its ability to activate transcription and that both of these activities are lost in a construct where residues within the second leucine zipper domain are eliminated.

Since transcriptional activation using the lexA reporter system requires a fusion partner that provides both an NLS and transactivation activity, the construct truncated at position 714 may be defective in one or the other or both of these functions. To distinguish among these alternatives, we added a heterologous NLS to the lexA-*rpm2*-Δ*C*(−*20*) construct. Figure 4D shows that the ectopic NLS does not improve the ability of lexA-*rpm2*-Δ*C*(−*20*) to activate transcription from the *HIS3* reporter gene. Thus, sequences between residues 715 and 735 of Rpm2p are necessary for transcriptional activation.

To determine the minimal domain for transcriptional activation, a set of N-terminal deletion mutant constructs, each designed to truncate Rpm2p by an average of 50 amino acids, fused to lexA were made and tested for their activities on the *HIS3* reporter (Fig. 5A and B). However, this analysis was hampered by the apparent instability of many fusion proteins. Deletion of the amino-terminal 520 residues had no effect on transcriptional activation by Rpm2p (Fig. 5B). Further deletion to amino acid 591, which removes the first leucine zipper, resulted in diminished but detectable protein levels; however, transcriptional activation was totally lost. Deletion to amino acid 684 resulted in a stable fusion protein, which retains a portion of Rpm2p including the second leucine zipper. However, transcriptional activity of this mutant was equal to that observed for lexA alone. Therefore, together these data indicate that the region for transcriptional activation lies between amino acids 521 and 735 of Rpm2p and contains two putative leucine zippers.

Rpm2p localizes lexA to both the nucleus and mitochondria. It is clear that Rpm2p can reach the nucleus if a portion of the mitochondrial leader sequence is missing. However, if the nuclear location is physiologically meaningful, Rpm2p must localize to both the nucleus and the mitochondria. To determine whether some Rpm2p localizes to the nucleus when the mitochondrial leader sequence is intact, we inserted a lexA coding sequence downstream of the mitochondrial leader sequence in wild-type *RPM2* and in the mutant alleles *rpm2-100*, *rpm2*-Δ*C*, and *rpm2*-Δ*C*(−*20*) (Fig. 6A). We used lexA fused to the Rpm2p mitochondrial leader sequence alone, MLS-lexA, as a control (Fig. 6A). We introduced these alleles on low-copy-number plasmids into cells carrying a wild-type *RPM2* gene on a *URA3*-containing plasmid and then measured the ability of these cells to grow on medium containing 5-FOA. Figure 6B demonstrates that cells with alleles harboring coding regions of either full-length *RPM2*, *rpm2-100*, or *rpm2*-Δ*C*, but not *rpm2*-Δ*C*(−*20*), can grow in the absence of wild-type *RPM2* on glucose. The latter has a truncated second leucine zipper.

Cells expressing these proteins from low-copy-number plasmids as their only source of *RPM2* (Fig. 6D) grow on rich glucose-containing medium (YPD) (Fig. 6C). Rpm2-lexA protein supports mitochondrial protein synthesis since cells grow like the wild type on nonfermentable carbon sources (Fig. 6C). Cells expressing rpm2-100-lexA fusion protein grow poorly on glycerol-ethanol-containing medium (Fig. 6C). This is not a surprise because the *rpm2-100* mutation itself gives the same phenotype (64). Cells expressing the C-terminal deletion mutation as an Rpm2-lexA fusion protein grow on glucose, but the cells lose their wild-type mitochondrial genome and are unable to grow by respiration, which is consistent with our published results (32, 63). Together, these results indicate that lexA does not interfere with any Rpm2p function(s).

To determine whether these fusion proteins function in the nucleus, we tested for the lexA operator-dependent activation of a *HIS3* reporter gene in the L40 strain as described above. Figure 7 demonstrates that cells expressing either MLS-lexA-Rpm2 or MLS-lexA-Rpm2-ΔC protein can grow in the absence of histidine and in the presence of AT, a competitive inhibitor of the *HIS3* gene product. Neither MLS-lexA-rpm2-100 nor MLS-rpm2-ΔC(−20) fusion protein can support growth in the absence of histidine. Thus, the *rpm2-100*-encoded mutant protein, which causes a translational defect of mitochondrion-encoded subunits of cytochrome *c* oxidase, loss of growth in the absence of the wild-type mitochondrial genome, and the ability to suppress the temperature-sensitive *tom40-3* mutant (64), also abolished its ability to activate transcription as a lexA fusion protein. Unlike the *rpm2* null mutation, the *rpm2-100* mutation abrogates a specific function of *RPM2*, suggesting that the lack of transcriptional activation by rpm2-100-lexA fusion protein is due to the loss of a physiologically relevant function.

Suppression of *tom40-3* temperature-sensitive growth correlates with the efficiency of transcriptional activation. Another very interesting observation from the previous experiment is that the C-terminally truncated protein has higher activity in

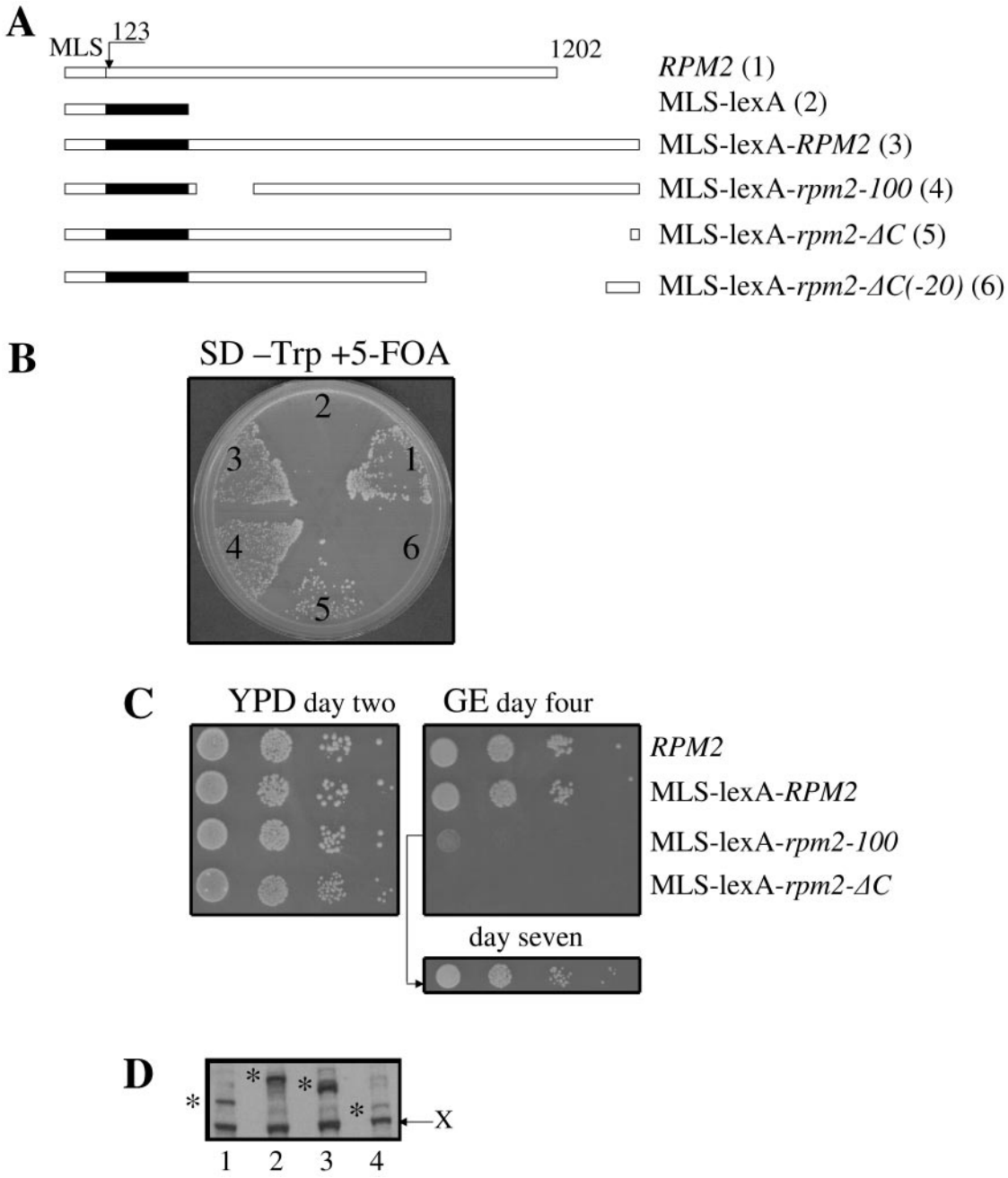

FIG. 6. (A) Schematic drawing of *RPM2* genes containing lexA between the mitochondrial leader sequence and the mature protein. The constructs are described in the text. (B) Growth on SD medium lacking tryptophan and containing 5-FOA. Numbers correspond to those following the construct names in panel A. (C) Cells from plate B were diluted 10-fold subsequently four times and spotted on glucose (YPD)- and glycerol-ethanol (GE)-containing plates and incubated as indicated in the figure. (D) Western analysis showing the fusion proteins marked with asterisks. Lanes: 1, Rpm2p; 2, MLS-lexA-Rpm2p; 3, MLS-lexA-rpm2-100p; 4, MLS-lexA-rpm2-ΔCp. The migration of an unknown yeast protein which is recognized by lexA antibodies serves as a loading control (X).

transactivation compared to full-length Rpm2p when expressed as a lexA fusion protein. Cells expressing Rpm2-ΔC-lexA fusion protein can grow in the presence of 5 mM AT, while cells with Rpm2-lexA do not. The allele specificity of *RPM2-lexA* transcriptional activation suggested that this property could be utilized to determine other activities associated with *RPM2* function. Multiple copies of *RPM2*, but not *rpm2-100*, can suppress a mutation in *tom40-3*, which encodes a temperature-sensitive component of the mitochondrial import channel (32, 64). Therefore, we asked whether the degree of suppression of a *tom40-3* mutant by different Rpm2p alleles correlates with transcriptional activation by the same Rpm2p alleles expressed as fusion proteins with lexA. To address this question, we transformed *tom40-3* mutant cells with *RPM2* and

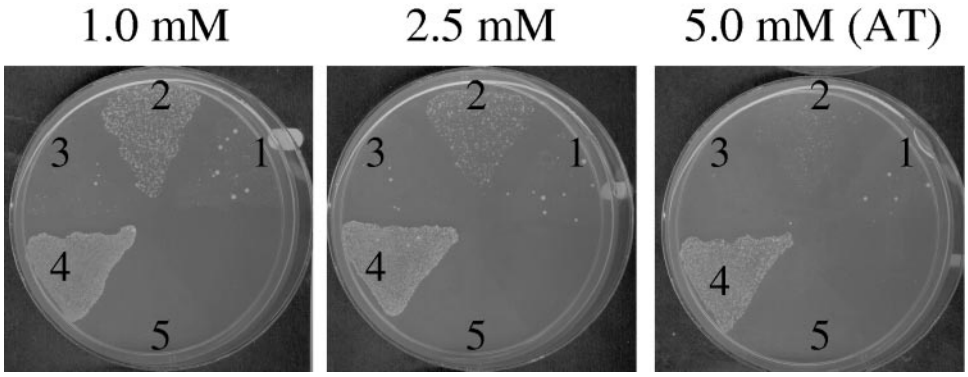

FIG. 7. Growth of MLS-lexA (strain 1)-, MLS-lexA-*RPM2* (strain 2)-, MLS-lexA-*rpm2-100* (strain 3)-, MLS-lexA-*rpm2*-Δ*C* (strain 4)-, and MLS-*rpm2*-Δ*C*(−*20*) (strain 5)-transformed cells in the absence of histidine and the presence of AT.

rpm2-Δ*C* alleles on either low- or high-copy-number plasmids and determined their ability to suppress *tom40-3* temperature-sensitive growth. Figure 8 demonstrates that the temperature-sensitive growth of *tom40-3* mutant cells is suppressed by the *rpm2*-Δ*C* allele on a low-copy-number plasmid. In contrast, comparable suppression by wild-type *RPM2* requires multiple copies. Thus, the ability of *RPM2* to suppress the *tom40-3* mutation correlates with its ability to support transcription.

Effect of Rpm2p on nucleus-encoded mitochondrial components in ρ° cells. The fact that Rpm2p is clearly capable of localizing to both mitochondria and the nucleus favors a model where Rpm2p influences the synthesis of nucleus-encoded proteins involved in mitochondrial biogenesis and function. This model predicts that the levels of Rpm2p targets (directly or indirectly) will differ in the *RPM2* and Δ*rpm2* backgrounds. To test this prediction, we started with a Δ*rpm2* strain that is viable because it carries the Δ*rpm2* multicopy suppressor *SEF1* (23). *SEF1* (suppressor of essential function) is a bypass suppressor of the essential function of *RPM2* but not the RNase P function (23). Consequently, these cells lose their wild-type mitochondrial genomes. We transformed this strain with vector alone or with *RPM2* and compared the steady-state levels of mRNAs coding for TOM complex components, the essential mitochondrial chaperones *HSP10* and *HSP60*, and *ATP2* mRNAs. In addition, we included wild-type strains with ([*rho*$^+$]) and without ([*rho*^{0}]) the wild-type mitochondrial genome as controls, since the loss of mtDNA itself induces changes in nuclear gene expression (10). Figure 9A demonstrates that the steady-state level of nucleus-encoded ATPase subunit β, *ATP2*, mRNAs is slightly reduced in cells without mtDNA compared to wild-type cells. However, this level remains comparable in the presence or absence of *RPM2* and does not appear to be influenced by overexpression of *SEF1*. Therefore, we used the *ATP2* mRNA as the normalization control in our real-time PCR experiments. After normalization to *ATP2*, we found that steady-state levels of mRNA encoding the multisubunit TOM complex components are increased in cells lacking mtDNA (compare strains 1 and 2 in Fig. 9B). These are the mRNAs encoding the core components Tom40p and Tom6p, as well as Tom20p-Tom22p and Tom37p-Tom70p, all components of two-receptor systems (2, 31, 32, 46, 49). In addition, mRNA levels of two essential mitochondrial chaperones, Hsp10p and

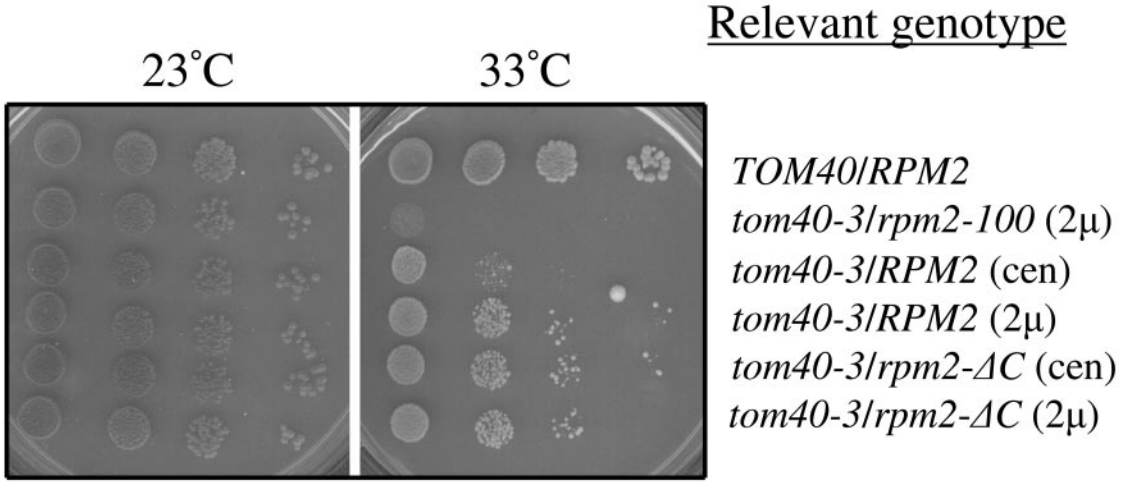

FIG. 8. Suppression of *tom40-3* temperature-sensitive growth by various *RPM2* constructs. Either a single-copy (cen) or a multicopy (2μm) plasmid coding for Rpm2p, rpm2-100p, or rpm2-ΔCp was introduced into cells carrying the *tom40-3* temperature-sensitive allele. Transformants were spotted in 1:10 serial dilutions on YPD plates together with the wild-type strain (*TOM40*).

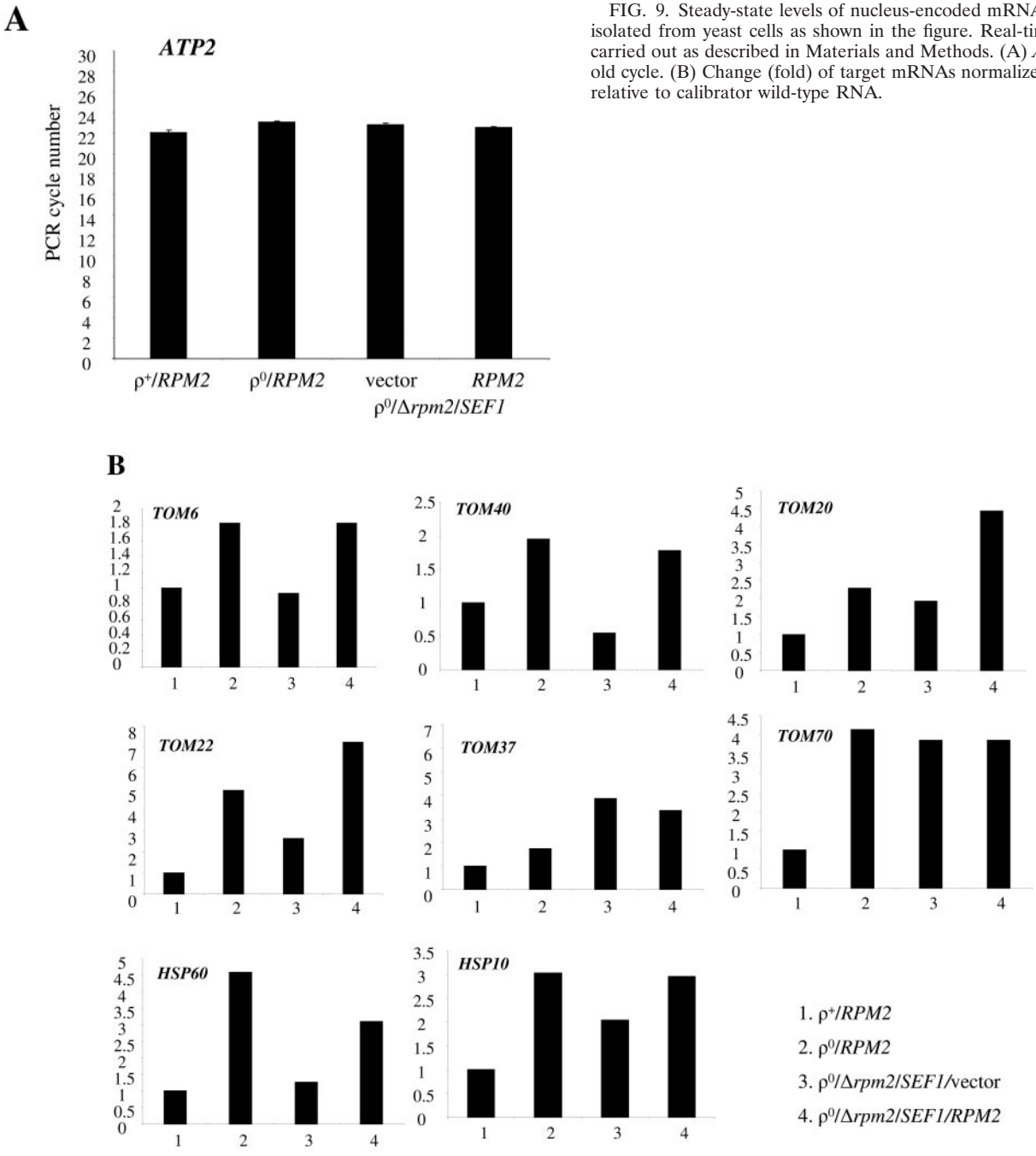

FIG. 9. Steady-state levels of nucleus-encoded mRNAs. RNA was isolated from yeast cells as shown in the figure. Real-time PCR was carried out as described in Materials and Methods. (A) *ATP2* threshold cycle. (B) Change (fold) of target mRNAs normalized with *ATP2* relative to calibrator wild-type RNA.

Hsp60p, are higher in [*rho*^{0}] cells. The increase of *TOM6*, *TOM20*, *HSP60*, and *HSP10* mRNAs in [*rho*^{0}] cells has been reported previously (66); however, the mechanism responsible for the observed changes is unknown. We demonstrate here first that the increase of *TOM6*, *TOM40*, and *HSP60* mRNAs in [*rho*^{0}] cells is *RPM2* dependent and is not affected by overexpression of *SEF1*. Second, *TOM20*, *TOM22*, *TOM37*, and *HSP10* mRNAs increase in [*rho*^{0}] cells and also show an increase compared to [*rho*$^+$] cells when there is overexpression of *SEF1*. Third, the levels of *TOM20*, *TOM22*, and *HSP10* but not *TOM37* further increase in cells overexpressing *SEF1* and carrying *RPM2* on a low-copy-number vector, suggesting an additive effect of both genes on certain TOM mRNAs. Fourth, expression of *TOM70* mRNA is similar in all [*rho*^{0}] cells (compare strains 2, 3, and 4 in Fig. 9B) and is independent of the overexpression of *SEF1* and additional copies of *RPM2*. These results indicate that Rpm2p, as well as its bypass suppressor *SEF1*, can affect certain components of the TOM import machinery and essential mitochondrial chaperons in cells without mtDNA.

Nucleus-encoded mitochondrial proteins Tom40p and Hsp60p, but not Atp2p, increase in the presence of *RPM2*. We

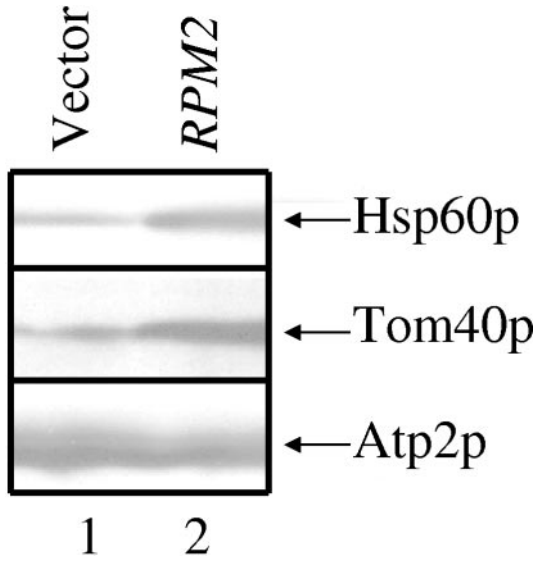

FIG. 10. Steady-state levels of some mitochondrial proteins increase in the presence of *RPM2*. Total protein was prepared from *SEF1*/Δ*rpm2*/vector (lane 1) and *SEF1*/Δ*rpm2*/*RPM2* (lane 2) cells and analyzed by Western blotting with antibodies that recognize nucleus-encoded mitochondrial proteins Hsp60p, Tom40p, and Atp2p.

performed Western analysis with proteins isolated from cells without mtDNA and either with or without *RPM2* to determine whether protein levels change in the same way that the mRNA levels changed. Western analysis was performed on cell extracts from strains 3 and 4 (Fig. 9B). Figure 10 demonstrates a significant reduction in essential protein Tom40p (2) and Hsp60p (15) but not Atp2p (13) levels in cells lacking *RPM2*. Therefore, we conclude that there is a specific, rather than a general, effect of *RPM2* on the steady-state level of mitochondrial proteins.

DISCUSSION

The yeast *S. cerevisiae* Rpm2 protein was originally identified as a component of mitochondrial RNase P (45). We demonstrate here that Rpm2p also localizes to the nucleus and *RPM2* constructs with a heterologous DNA-binding domain inserted downstream of the mitochondrial targeting signal support mitochondrial RNase P activity and activate transcription of a reporter gene in the nucleus. Therefore, Rpm2p has information for mitochondrial, as well as nuclear, localization. In addition, in cells with dysfunctional mitochondria, Rpm2p affects the levels of mRNAs encoding certain components of TOM complexes, as well as mitochondrial heat shock proteins.

Targeting and translocation of most nucleus-encoded mitochondrial proteins depend on N-terminal extensions referred to as mitochondrial leader sequences or presequences (45, 49). With a few exceptions, a presequence typically consists of about 15 to 40 amino acid residues, which is cleaved upon import into the organelle (5). The DNA sequence of *RPM2* predicts a protein of 1,202 amino acids with a calculated molecular mass of 139,347 Da. Although we have never shown directly that the first 122 amino acids of Rpm2p are sufficient for directing a passenger protein into the organelle, it is clear that information for targeting is present in these first 122 amino acids. First, this peptide shares sequence features common to mitochondrial targeting signals such as the absence of acidic residues and the presence of basic residues and hydroxylated residues (49). Second, this sequence has a motif which is found in precursors that are cleaved in one step by mitochondrial processing peptidase (5). Third, the amino terminus of Rpm2p obtained by direct protein sequencing starts with amino acid 123 of the deduced protein sequence (45). Fourth, we show here that the lexA-Rpm2p fusion protein missing the first 43 amino acids provides the essential function but not the mitochondrial functions, suggesting that the non-RNase P function of Rpm2p is outside the mitochondria.

Studies with GFP revealed that amino acids 44 to 735 are sufficient to localize a GFP-Rpm2 fusion protein to the nucleus, which indicates that nuclear localization of the fusion protein is dependent on Rpm2p. Huh et al. (28) observed mitochondrial staining but not nuclear staining in their studies. We know from our own studies that the essential function phenotype is expressed even though Rpm2p protein cannot be detected by Western blot assays, suggesting that Rpm2p levels in nuclei are low. However, using another reporter gene assay we found that lexA-Rpm2 fusion protein missing the first 43 amino acids activates transcription of promoters containing lexA-binding sites, indicating that Rpm2p can be localized to the nucleus and act as a transcriptional activator. We also demonstrate here that Rpm2p localizes to both mitochondria and the nucleus in the presence of a mitochondrial leader sequence. Proteins larger than 60 kDa require active transport into the nucleus, but further work is necessary to understand the details of how nuclear localization is promoted.

Rpm2p, like many other transcriptional activators, contains two putative leucine zipper domains, at amino acid residues 527 to 555 and 696 to 717. A deletion mutation that eliminated two amino acids at the end of the second zipper domain abolished the essential function of Rpm2p without discernibly affecting protein stability (63). We demonstrate here that this domain is also critical for transcriptional activation. Furthermore, deletion mapping revealed that the region required for transcriptional activation lies between amino acids 521 and 735 of Rpm2p and requires both leucine zippers.

Unlike the *rpm2* null mutation, another *RPM2* mutation, *rpm2-100*, abrogates a specific function of *RPM2*. The function of *RPM2* compromised by the *rpm2-100* mutation makes yeast cells dependent on the wild-type mitochondrial genome for fermentative growth (64), indicating that Rpm2p is essential in cells with dysfunctional mitochondria. Other proteins required for respiratory growth also become essential for growth on fermentable carbon sources in the absence of wild-type mtDNA. These genes include *AAC2*, encoding the ADP/ATP translocase (36); *PGS1*, encoding the phosphatidylglycerol phosphate synthase (65); *YME1*, encoding the ATP-zinc-dependent mitochondrial protease (68); and genes encoding the α and β subunits of F1 ATPase (13). It is unclear why cells require wild-type mtDNA when these genes lose function, since the lack of function does not lead to an obvious common defect. Nonetheless, each of these genes can be tied, either directly or indirectly, to the mitochondrial import process (20, 35; for a review, see references 14 and 17). Indeed, there is direct evidence indicating that cells with defects in mitochon-

drial protein import depend on wild-type mtDNA for growth on fermentable carbon sources (20). Mutation of any of the six genes (*TIM18*, *TIM54*, *TIM10*, *TIM9*, *TIM12*, and *TOM70*) that function in the *TIM22* pathway (transport inner membrane) is incompatible with loss of mtDNA (20, 33, 57). Thus, it is possible that mutations in these genes, as well as *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 the point necessary to maintain the organelle. This notion is supported by the results of Lefebvre-Balguerie et al. (37) indicating that F1-catalyzed hydrolysis of ATP is essential for maintaining an electrochemical mitochondrial membrane potential and when it is compromised, mitochondrial Hsp60 precursors accumulate.

The observation that multiple copies of *RPM2* can suppress a mutation in a key component of the mitochondrial protein import channel (32) while *rpm2-100* cannot (64) suggests a model relating a non-RNase P function to mitochondrial import. Although the mechanism of suppression of *tom40-3* is unclear, we demonstrate here that increased levels of *TOM6*, *TOM40*, and *HSP60* mRNAs depend on Rpm2p function in cells lacking wild-type mtDNA. In addition, the *rpm2-100* mutation that causes a translational defect of mitochondrion-encoded subunits of cytochrome *c* oxidase and loss of growth in the absence of the wild-type mitochondrial genome (64) also abolishes the Rpm2p transcriptional activity of the Rpm2-lexA fusion protein. This suggests that the lack of transcriptional activation by rpm2-100-lexA protein is due to the loss of a physiologically relevant function. These observations lead to the model that Rpm2p affects transcription of nuclear genes and is required to maintain sufficient levels of nucleus-encoded components of the mitochondrial import machinery in cells with dysfunctional mitochondria. We found previously that reduced proteasome activity in *pre4-2* and *ump1-2* mutants allows growth in the absence of *RPM2* (41). This is also consistent with the model because decreased degradation could also lead to increases in components of the mitochondrial import apparatus.

Changes in the functional state of mitochondria cause changes in nuclear gene expression (47). These changes are, for the most part, adaptive in that they represent cellular adjustments to altered mitochondrial states (for a review, see reference 10). The status of mtDNA affects nuclear gene expression in both yeast and mammalian cells (1, 4, 21, 24, 38, 47, 51, 52, 66). This important phenomenon, called retrograde regulation, was first discovered in the yeast *S. cerevisiae* (38, 47, 53). Rtg1p, Rtg2p, and Rtg3p (29, 56) are the key components of the retrograde signaling pathway required for the expression of some retrograde responsive genes (16, 21, 40). One function of retrograde signaling is to maintain glutamate levels in cells with dysfunctional mitochondria (21, 40). However, in the presence of glutamine, disruption of the *RTG1-3* pathway does not prevent growth on either fermentable or nonfermentable carbon sources. Other genes that change expression in response to mitochondrial dysfunction do not appear to be under the control of the Rtg1-3 proteins. For example, a small number of retrograde-responsive genes that confer pleiotropic drug resistance are *RTG* independent and regulated by two closely related transcription factors, Pdr1p and Pdr3p (19, 24, 66). Other genes, however, might be regulated by additional RTG- and pleiotropic drug resistance-independent pathways involved in mitochondrion-to-nucleus signaling.

Four percent of yeast genes reproducibly alter transcription in yeast cells devoid of mtDNA when grown on glucose (66). Of these genes, 86% were induced between 1.5- and 6-fold while 14% were repressed. About one-fifth of the genes with elevated expression in cells lacking mtDNA encode proteins involved in mitochondrial biogenesis and function, indicating cellular accommodations to the mitochondrial defects. Previous work showed that *TOM6*, *TOM20*, *HSP10*, and *HSP60* are upregulated in cells lacking mtDNA (66), and we show here that this increase in cells lacking mtDNA depends on *RPM2*. Moreover, the effects of *SEF1* and *RPM2* appear additive in upregulation of *TOM20* and *TOM22*, while there is no effect of *RPM2* on *TOM37* and *TOM70*, although the latter mRNAs are induced in [*rho*^{0}] cells. We believe that induction of TOM components and the essential chaperones in cells lacking mtDNA is most likely an adaptation to maintain efficient protein import upon reduction in membrane potential caused by the loss of mtDNA. The transcriptional induction of mitochondrial chaperones also occurs in mammalian cells after depletion of mtDNA (43), and overexpression of Hsp10 and Hsp60 proteins inhibits myocardial apoptosis in response to ischemic injury (39) and doxorubicin-induced cardiomyopathy (58).

Our observation that Rpm2p affects expression of nucleus-encoded mitochondrial proteins in cells with dysfunctional mitochondria and activates transcription if brought to the DNA strongly implicates Rpm2p in transcription. Since Rpm2p clearly has RNA binding activity in its role as a subunit of mitochondrial RNase P, it may be that RNA binding activity is also important to its role in transcription. There also may be a regulatory significance to mitochondrial biogenesis because Rpm2p is both mitochondrial and nuclear and because Rpm2p is required to maintain normal levels of essential mitochondrial chaperones in cells with dysfunctional mitochondria. In either case, it is clear that Rpm2p is a strong candidate for a regulatory protein intimately involved in mitochondrial biogenesis and critical to maintaining viability when cells lose their mitochondrial genome. Therefore, Rpm2p provides a vehicle to further our understanding of mitochondrial biogenesis and the complex regulatory networks necessary to the organization and maintenance of this critical and essential organelle.

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