Rmd9p Controls the Processing/Stability of Mitochondrial mRNAs and Its Overexpression Compensates for a Partial Deficiency of Oxa1p in *Saccharomyces cerevisiae*

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ABSTRACT

Oxa1p is a key component of the general membrane insertion machinery of eukaryotic respiratory complex subunits encoded by the mitochondrial genome. In this study, we have generated a respiratory-deficient mutant, *oxa1-E65G-F229S*, that contains two substitutions in the predicted intermembrane space domain of Oxa1p. The respiratory deficiency due to this mutation is compensated for by overexpressing *RMD9*. We show that Rmd9p is an extrinsic membrane protein facing the matrix side of the mitochondrial inner membrane. Its deletion leads to a pleiotropic effect on respiratory complex biogenesis. The steady-state level of all the mitochondrial mRNAs encoding respiratory complex subunits is strongly reduced in the Δrmd9 mutant, and there is a slight decrease in the accumulation of two RNAs encoding components of the small subunit of the mitochondrial ribosome. Overexpressing *RMD9* leads to an increase in the steady-state level of mitochondrial RNAs, and we discuss how this increase could suppress the *oxa1* mutations and compensate for the membrane insertion defect of the subunits encoded by these mRNAs.

THE biogenesis of respiratory complexes is an intricate process that requires the co-assembly of mitochondrial and nuclear-encoded subunits with prosthetic groups that are essential to the electron transport activity. This process needs the assistance of nuclear-encoded proteins that control various steps of mitochondrial gene expression, such as mRNA processing/stability, translation, and the final assembly of the various subunits with the prosthetic groups (Tzagoloff and Dieckmann 1990; Grivell *et al.* 1999). Most of these factors control the expression or assembly of a single subunit or cofactor, and the deletion of the corresponding gene leads to a defect specific to a given respiratory complex (e.g., for cytochrome oxidase assembly; Fox 1996; Barriontos 2002; Herrmann and Funés 2005; Khalimonchuk and Rödel 2005).

One of these nuclear-encoded factors is Oxa1p, which is a key component of the general membrane insertion machinery of respiratory complex subunits. In the yeast *Saccharomyces cerevisiae*, the deletion of *OXA1* is viable but impairs the biogenesis of the three respiratory complexes of dual genetic origin (complexes III, IV, and V) and results in the degradation of the membrane subunits (Bauer *et al.* 1994; Bonnefoy *et al.* 1994; Altamura *et al.* 1996; Lemaire *et al.* 2000). Oxa1p is required for translocation of the hydrophilic domain of the complex IV subunit Cox2p (He and Fox 1997; Hell *et al.* 1998; Herrmann and Bonnefoy 2004). It interacts with nascent mitochondrial-encoded polypeptides (Hell *et al.* 1998, 2001) and its C-terminal tail, located in the matrix, binds to the mitochondrial ribosome (Jia *et al.* 2003; Szyrach *et al.* 2003; Ott *et al.* 2006). Thus, Oxa1p participates in the cotranslational insertion of mitochondrial-encoded subunits of the three respiratory complexes from dual genetic origin into the mitochondrial inner membrane.

Oxa1p is functionally conserved in prokaryotes and eukaryotes. In filamentous fungi, Oxa1p is required for the assembly of complexes I, III, IV, and V and is essential for viability in these strictly aerobic organisms (Nargang *et al.* 2002; Sellem *et al.* 2005). In *Escherichia coli*, depletion of the Oxa1 homolog, YidC, leads to a defect in the assembly of cytochrome o oxidase and ATP synthase (van der Laan *et al.* 2003; for review Dalbey and Kuhn 2004) and affects the translocation of the Cox2p homolog, the CyoA subunit (Celera *et al.* 2006; van Bloois *et al.* 2006). The mitochondrial Oxa1p forms a homo-oligomer (Nargang *et al.* 2002) while a molecular interaction with the SEC translocase has been described in *E. coli* (Nouwen and Driessen 2002).

The mitochondrial Oxa1p is composed of three domains located in the intermembrane space, the inner membrane, and the matrix. To understand the precise role of these domains, we have constructed point and deletion mutants (Lemaire *et al.* 2004 and this work).
and searched for suppressor genes able to compensate for the respiratory deficiency due to these various oxa1 mutations. This strategy led to the identification of genetic and high-copy suppressors. First, missense mutations in the transmembrane domain of two subunits of the respiratory complex III, Cyt1p and Qcr9p, are able to compensate for the absence of Oxa1p (Hamel et al. 1998; Saint-Georges et al. 2001). Second, two high-copy suppressors, OMS1 and HAP4, suppress only some missense oxa1 alleles (Lehail et al. 2004; Hlavacek et al. 2005). Thus there are various ways of bypassing or compensating for oxa1 defects.

In this study, we generated the oxa1-E65G-F229S mutant that harbored a double amino acid substitution in the intermembrane space domain and isolated the RMD9 gene as a high-copy suppressor of this mutation. We show that RMD9 encodes an extrinsic membrane protein facing the matrix side of the mitochondrial inner membrane. An analysis of mitochondrial mRNA and rRNA accumulation in the Δrmd9 mutant reveals defects in the processing/stability of all the mRNAs that encode membrane subunits of respiratory complexes and, to a lesser extent, in the accumulation of two RNAs encoding components of the small ribosome subunit. In addition, overexpression of RMD9 leads to an increase in the steady-state level of mitochondrial mRNAs. Hypotheses of how this increase could compensate for the membrane insertion defect due to some oxa1 mutations are presented.

**MATERIALS AND METHODS**

Strains, media, and genetic techniques: All the strains are derived from the W303 nuclear background MATA ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100. CW04 and CW252 are wild-type strains and carry an intron-plus mitochondrial genome or an intron-less mitochondrial genome, respectively. In the intron-less mitochondrial genome (Seraphin et al. 1987), a cytochrome b mutation (cytb-G252D) was originally present. This mutation, leading to a slight thermosensitive respiratory phenotype, has been corrected in the strain CW252 (Saint-Georges et al. 2002). NBT1 carries the oxa1::LEU2 allele (Bonnefoy et al. 1994).

The maintenance of the mitochondrial genome in the rmd9::GA18 strain was regularly verified by crossing the rmd9::GA18 cells with the RMD9-strain KL14-4A/60 devoid of the mitochondrial genome (MATA his1, top2, rho0) and testing the growth of the diploids on glycerol medium.

Most media and genetic techniques were as described in Dujardin et al. (1980). The nonfermentable media contain either glycerol 2% or ethanol and glycerol (2% each). The fermentable media contain either glucose 2% or galactose 2% and glucose 0.1%.

Generation of the oxa1-E65G-F229S mutant by random PCR mutagenesis: The OXA1 gene was amplified using the low fidelity Taq polymerase (Advantage 2, CLONTECH, Palo Alto, CA). The PCR fragments were cloned in the centromeric plasmid pNB160 (Lehail et al. 2004), and the oxa1::LEU2 strain (NBT1) was transformed with the resulting plasmids. The respiratory phenotype of the transformants was tested on glycerol/ethanol media at 28° and 36°. Plasmids were recovered from transformants exhibiting a thermosensitive respiratory deficiency, and the gene was sequenced to identify the nature of the mutation(s) responsible for the defect. The oxa1-E65G-F229S mutant contains two substitutions in distant codons: GAA → GGA at codon 65 and TTC → TCC at codon 229. The mutated oxa1-E65G-F229S gene was integrated into the genome by homologous recombination. The respiratory phenotype of the integrated mutant was similar to that of the oxa1::LEU2 strain expressing this mutation from a centromeric plasmid.

**High-copy suppressor isolation:** The oxa1-E65G-F229S cells were transformed with a high-copy library made in the URA3 2μ vector pFL44L (Bonneau et al. 1991). [URA+] clones were selected and replica plated onto glycerol medium at 36°. Total yeast genomic DNA was extracted from cosegregating slow-growing glycerol-positive clones and used to transform E. coli cells to recover the plasmids. Molecular analysis by restriction enzymes and sequencing allowed the identification of the chromosomal fragment present in each plasmid.

RMD9 disruption and epitope tagging of Rmd9p and Ybr238p: The RMD9 gene was inactivated in the haploid strain CW252, using the PCR inactivation method described by Wach et al. (1994): the entire ORF was replaced by the Kan marker gene that confers resistance to the G418 drug.

Rmd9p and Ybr238p were tagged at their C terminus with c-Myc and HA epitopes, respectively, using the S. cerevisiae pombe HIS1 marker gene (which complements the S. cerevisiae his3 mutation) as described in Longtine et al. (1998). The PCR fragments were used to transform the wild-type strain (CW252) to histidine prototrophy. Correct integrations of the tags at the RMD9 or YBR238 locus were confirmed by PCR amplification and sequencing.

**Mitochondria isolation and immunoblotting:** Mitochondria were isolated by differential centrifugations after digestion of cell walls with Zymolase-100T (Kermorgant et al. 1997). The protein concentration of the final mitochondrial suspension was determined using the Bio-Rad (Hercules, CA) assay. Mitochondrial proteins were resolved on SDS-polyacrylamide gels followed by immunoblotting (reinforced nitrocellulose, Schleicher & Schuell, Keene, NH). Polyclonal antibodies against cytochrome c1 or cytochrome b were raised against a fusion proA-apocytochrome c1 expressed in E. coli or a synthetic peptide of Cytb coupled to KLH (AGRO-BIO), respectively. Monoclonal antibodies against Cox2p, porin, and PGK were from Molecular Probes (Eugene, OR) and the anti-HA was from Santa Cruz Biotechnology. Other antibodies were gifts: Cytb2p (B. Guirard, Gif-sur-Yvette, France), Atp6p (J. Velours, Bordeaux, France), Arg8p (T. D. Fox, Ithaca, NY), and c-Myc (J. M. Galan, Paris). Bound antibodies were detected by horseradish-peroxidase-conjugated secondary antibodies (Promega, Madison, WI). Proteins were visualized using chemiluminescent substrate (Pierce Chemical, Rockford, IL). Protein markers (Precision Plus protein standards, Dual Color Bio-Rad) were used to estimate protein molecular weights.

**Cytochrome absorption spectra:** Cytochrome absorption spectra of whole cells were recorded at liquid nitrogen temperature after reduction by dithionite using a Cary 400 (Varian, San Fernando, CA) spectrophotometer. Cytochromes c1 and b are part of complex III while cytochromes a + a3 are part of complex IV. Absorption maxima for the α-hands of cytochromes c1, b, and a + a3 are expected at 546, 552, 558, and 602 nm, respectively.

**RNA extraction and RNA hybridization:** Cells were harvested at exponential growth phase and total RNAs were purified by the “hot phenol” technique (Rakic et al. 2000). The amount of RNAs was estimated by spectrophotometer measurement at 260 nm. The RNAs were separated on 1.2% agarose formaldehyde gels and were transferred on Hybond-C
extra membrane (Amersham, Buckinghamshire, UK). Prehybridization and hybridization were done at 42° in 50% formamide in the presence of Denhardt. For detection of the mitochondrial RNAs, the following PCR-amplified fragments internal to each mitochondrial gene were generated and used to produce radiolabeled probes by random priming (random primer DNA labeling system from Invitrogen, San Diego): for COX1, 1.6 kb; COX2, 0.75 kb; COX3, 0.81 kb; CYTB, 0.65 kb; ATP6/8, 0.7 kb; ATP9, 0.25 kb; VAR1, 0.63 kb; J5S, 0.8 kb; 21S, 0.8 kb; rRNA6S, 0.8 kb; ACT1N, 1.1 kb; RMD9, 0.8 kb; and CYTI, 0.6 kb.

RESULTS

New mutations in the intermembrane space domain of Oxa1p affect respiratory complex assembly: To investigate the function of the different domains of Oxa1p, we used a PCR-based random-mutagenesis strategy to generate point mutations, which were subsequently integrated at the chromosomal OXA1 locus (see MATERIAL AND METHODS). We obtained the oxa1-E65G-F229S mutant that harbored a double amino acid substitution in a domain predicted to reside in the intermembrane space (Figure 1A) and presented a clear thermosensitive respiratory deficiency (Figure 1B). The oxa1-E65G-F229S mutations led to a strong decrease of peaks corresponding to cytochrome b and a3 when cells were grown at 36°C (Figure 1C) and of the steady-state level of mitochondrial-encoded subunits of complexes III, IV, and V (data not shown). Thus the oxa1-E65G-F229S mutations conferred a pleiotropic effect on the assembly of respiratory complexes at 36°C.

The respiratory deficiency of the oxa1-E65G-F229S mutant can be compensated for by the overexpression of the RMD9 gene: In the search for genetic interactions involving the OXA1 gene, we looked for high-copy suppressors able to alleviate the respiratory defect due to the oxa1-E65G-F229S mutations. A genomic library cloned in a high-copy vector was introduced into the oxa1 cells. Transformants able to restore a slow growth on nonfermentable medium at 28°C and 36°C were further analyzed. The fast-growing transformants were not selected as we reasoned that they might contain the OXA1 gene. One selected transformant contained a plasmid carrying a 4.6-kb fragment of chromosome VII (Figure 2A) that still conferred suppression after retransformation of the oxa1 mutant (Figure 2B). This genomic fragment contained four entire ORFs: YGL106w/MLC1, YGL107c/RMD9, YGL108c, and YGL109w. Subcloning experiments showed that the gene responsible for the suppression was YGL107c/RMD9. Overexpression of RMD9 in the oxa1-E65G-F229S mutant restored ~70% of cytochrome assembly as shown by cytochrome spectra analysis (Figure 2C).

The specificity of action of RMD9 overexpression was tested on four oxa1 mutations that have been previously described (see Figure 1A): (1) the Δoxa1 null allele (Bonnefoy et al. 1994), (2) the oxa1-L240S mutation (ts1402; Meyer et al. 1997), (3) the oxa1-WW128AA double mutations, and (4) the oxa1-DΔL1-K332stop double mutations (Lemaire et al. 2004). Each mutant was transformed with the high-copy plasmid carrying RMD9 and the transformants were tested for their ability to grow on nonfermentable medium at 28°C and 36°C. Overexpression of RMD9 restored only very slow growth in the oxa1-L240S mutant (data not shown). Thus, RMD9 appears to be a high-copy suppressor specific for oxa1 mutations, affecting residues predicted to be located in the intermembrane space.
Deletion of RMD9 leads to pleiotropic effects on respiratory complex biogenesis: The RMD9 gene was previously isolated in a search for genes required for meiotic division (Enyenih et al. 2003), and systematic deletion analysis suggested that it could play a role in respiratory growth (Dimmer et al. 2002). To further investigate its role, a Δrmd9 null allele was constructed in the haploid strain CW252 (see Materials and Methods) containing an intron-less mitochondrial genome; this is often more stable than the intron-plus mitochondrial genome in respiratory-deficient mutants (e.g., Groudinsky et al. 1993; Rouillard et al. 1996). The null mutant exhibited a clear respiratory growth defect on nonfermentable substrate such as glycerol (Figure 3A). To determine the fate of mitochondrial DNA (mtDNA) in the mutant, Δrmd9 cells were crossed with a tester RMD9 rho0 strain, devoid of mitochondrial genome, and the respiratory capacity of diploids was analyzed. Twenty to 50% of diploids were respiratory deficient when the parental Δrmd9 cells were grown on glucose-rich medium before the cross; this amount was reduced to <10% when cells were grown on minimal medium. Thus, the absence of Rmd9p leads to some mtDNA instability on glucose-rich medium. However, the absence of Rmd9p leads to the rapid loss of mtDNA in the strains whose mtDNA contains introns (Williams et al. 2007, this issue; data not shown).

The effect of the absence of Rmd9p on respiratory complex biogenesis was studied by recording cytochrome spectra and analyzing mitochondrial protein accumulation. A cross with a tester rho0 strain ensured that these biochemical characterizations were done on cell cultures composed of at least 95% of rho+ cells. Cytochrome spectra showed a defect in cytochrome b and a+-assembly (Figure 3B). Interestingly, it was similar to the cytochrome spectra observed in the Δoxa1 mutant (Bonnefoix et al. 1994). In addition, the steady-state levels of the mitochondrial-encoded subunits Cox2p, Cytb, and Atp6p, which are representative of the three respiratory complexes of dual genetic origin, were drastically diminished, compared to the nucleaus-encoded CytL1 (Figure 3C).

These results show that the respiratory deficiency observed in the absence of Rmd9p is due to a defect in the biogenesis of respiratory complexes III, IV, and V.

Rmd9p is an extrinsic membrane protein facing the mitochondrial matrix: Large-scale analysis of the localization of S. cerevisiae proteins suggests that Rmd9p is localized in mitochondria (Huh et al. 2003; Sickmann et al. 2003). To verify its subcellular localization, Rmd9p was tagged at its C terminus with three c-Myc epitopes. We ensured that cells expressing the Rmd9p-c-Myc protein did not exhibit any respiratory phenotype (data not shown). The presence of Rmd9p-c-Myc in purified mitochondria and postmitochondrial supernatant was analyzed by immunodetection. The c-Myc antibody revealed a protein of the expected size, ∼90 kDa, that

Figure 2.—Restoration of respiratory complexes assembly by overexpressing RMD9 in the oxa1-E65G-F229S mutant. (A) The arrows represent the direction of transcription of the different ORFs in the region of chromosome VII carrying the suppressor gene. The genomic fragments inserted in the plasmids are indicated by thin straight lines. YepSU27 and YepSU28 were obtained after transformation of the oxa1-E65G-F229S mutant with the high-copy library in pFL44L (see Materials and Methods). The plasmid YepSU29 carrying only RMD9 was obtained after PCR amplification from YepSU27 and cloning into pFL44L. The mutant cells were transformed with these different plasmids and the respiratory growth of the transformants was tested. +, growth on nonfermentable substrate. (B) The oxa1-E65G-F229S mutant was transformed with three different high-copy plasmids: YepSU27 carrying RMD9 (RMD9, RMD9 overexpression), the empty control vector pFL44L, and YepNB6 carrying OXA1 (OXA1). The transformants were patched onto minimal glucose medium lacking uracil (glucose) and replica plated on nonfermentable medium (ethanol/glycerol). The plates were incubated for 4 days at 36°C. (C) Cytochrome absorption spectra of the oxa1-E65G-F229S cells transformed with the high-copy plasmids YepNB6 (OXA1) or YepSU27 (RMD9), grown on ethanol/glycerol medium for 2 (OXA1) and 4 (RMD9) days at 36°C, were recorded as described in the legend of Figure 1.
was recovered in the mitochondrial fraction, as was the *bona fide* mitochondrial protein Arg8p, whereas the cytosolic phosphoglycerate kinase (PGKp) was found mainly in the postmitochondrial supernatant (Figure 4A). To test whether Rmd9p was associated with mitochondrial membranes, mitochondria purified from Rmd9p-c-Myc-expressing cells were either alkali treated or sonicated and the soluble and membrane fractions were analyzed by immunodetection. The Rmd9p-c-Myc protein was distributed between both the membrane and the soluble fractions after alkali treatment and was found in the membrane fraction after sonication (Figure 4B; data not shown). As expected, Cox2p, which is embedded within the inner membrane, was recovered only in the pellet fractions in the two experiments while the soluble Arg8p protein was found mostly in the supernatants. Thus, Rmd9p appears associated with the membrane. Finally, we performed swelling experiments to disrupt the outer membrane of mitochondria in the presence or absence of proteinase K. Figure 4C shows that Rmd9p-c-Myc was protected from the protease as was the matrix protein Arg8p whereas the intermembrane space protein Cytb2p was completely degraded. Altogether, our results indicate that Rmd9p is an extrinsic membrane protein facing the mitochondrial matrix compartment.

**Genetic interaction between RMD9 and YBR238:** BLAST searches reveal that Rmd9p is related to another *S. cerevisiae* protein, Ybr238p. The two proteins are, respectively, 646 and 732 amino acids long and share ~45% of amino acid identity. A systematic deletion analysis has shown that the Δybr238 mutant is viable (Winzeler *et al.* 1999). We studied the effect of deleting *YBR238* on respiratory complex biogenesis by analyzing the respiratory growth and recording cytochrome spectra at various temperatures. We were unable to detect any impairment of respiration, and the rho~/rho+ production was identical to that in the wild-type strain (data not shown).

Large-scale analysis of the *S. cerevisiae* protein localization suggests that Ybr238p could be localized in the cytoplasm and/or mitochondria (Hu *et al.* 2003). We epitope tagged Ybr238p at its C terminus with HA epitopes and found a tagged protein of the expected 75 kDa size in purified mitochondria (Figure 4A). Moreover, after an alkali treatment of purified mitochondria
from Ybr238p–HA-expressing cells, the protein was found in the pellet fraction (Figure 4B), showing that it is a membrane-bound protein although in silico analysis does not predict any transmembrane domain.

To test for genetic interactions between RMD9 and YBR238, we constructed a double mutant, Δybr238 Δrdm9. The double mutant was viable but respiratory deficient. Its mitochondrial genome was highly unstable whatever the growth medium, leading to the rapid accumulation of rho0 cells. Thus, the two mutations together had a much more deleterious effect than each single mutation on mtDNA stability. This genetic interaction could suggest a functional interaction between the two proteins Rmd9p and Ybr238p.

**Rmd9p controls the processing/stability of several mitochondrial RNAs:** We have shown that Rmd9p is a mitochondrial protein whose absence affects the biogenesis of respiratory complexes III, IV, and V of dual genetic origin. Given its localization, Rmd9p could affect transcription, mRNA stability, the translation of respiratory subunits that are encoded by the mtDNA, or the assembly of the various subunits into functional complexes.

To determine which step was controlled by Rmd9p, we compared the steady-state levels of mitochondrial transcripts in Δrdm9 and wild-type strains carrying the intron-less mitochondrial genome. As shown by a cross with a tester rho0 strain, the RNA extractions done on galactose-grown cells were composed of at least 95% of rho0 cells. Total RNAs isolated from the two strains were analyzed by hybridization experiments with nine different probes. First, we determined the steady-state levels of RNAs of the three mitoribosomal components encoded by the mtDNA: the large ribosomal 21S rRNA, the small ribosomal 15S rRNA, and the mRNA encoding the mitoribosomal protein Var1p (Figure 5A). In the absence of Rmd9p, the steady-state level of 21S rRNA was unchanged. The hybridization with the 15S rRNA probe revealed two RNAs, one corresponding to the expected size of the 15S rRNA and the other probably due to a partial degradation of the 15S rRNA. The VAR1 mRNA level was slightly diminished. Finally, we have shown that the steady-state level of tRNA16s was not affected by the Δrdm9 mutation. Thus, the absence of Rmd9p did not affect the accumulation of the large 21S rRNA and tRNA16s but lowered the steady-state levels of the 15S rRNA and VAR1 mRNA.

Second, we analyzed the impact of the Δrdm9 mutation on mRNAs encoding respiratory complex subunits (Figure 5B). In the absence of Rmd9p, the steady-state levels of mRNAs encoding the cytochrome b (CYTB), the three Cox subunits (COX1, -2, -3), and the three ATPase subunits (ATP6, -8, -9) were strongly reduced or even undetectable. COX1, ATP8, and ATP6 genes belong to the same transcription unit and the ATP9 gene is cotranscribed with the downstream gene VAR1 gene. Large and unidentified transcripts that are not processed cotranscripts but correspond to precursors of specific mRNAs were detected with the probes for COX1 or ATP6/8 or ATP9 mRNAs (Figure 5C). Moreover, the fact that the VAR1 mRNA level was only slightly affected in Δrdm9 showed that the transcription of this bicistronic unit did occur in the Δrdm9 mutant. The absence of a transcript of the correct size for ATP9 and the presence of large transcripts that are not revealed by the VAR1 probe suggest that Rmd9p could control the processing and/or stability of the ATP9 mRNA. Furthermore, the normal accumulation of the tRNA16s that is cotranscribed with CYTB mRNA offers an additional argument in favor of a role for Rmd9p at a post-transcriptional level.

In conclusion, the absence of Rmd9p leads to a pleiotropic effect on the steady-state levels of several mitochondrial transcripts with a strong decrease of all mRNAs encoding subunits of respiratory complexes and a slight effect on both 15S rRNA and VAR1 mRNA that encode components of the small subunit of the mitochondrial ribosome.

**Figure 5.—**Analysis of mitochondrial transcripts in the Δrdm9 mutant. Total RNAs were isolated from wild-type (WT, CW252) and Δrdm9 strains grown in galactose medium at 28° C. Isolation of total RNAs was repeated twice and each preparation was analyzed on several blots using the 0.5- to 10-kb RNA ladder as a size marker (Invitrogen). Equivalent amounts of 2 and 6 μg were analyzed by hybridization with various mitochondrial probes: (A) Genes encoding the mitoribosomal protein, Var1p, the two rRNAs 21S and 15S, the tRNA16s, and Cytlp as control. For the small tRNA16s, we checked that it was absent in the control rho0 strain. (B) Genes encoding respiratory complex subunits: CYTB, COX2, COX3, COX1, ATP6/8, ATP9, and the 21S rRNA as control. The positions of size markers are indicated at the right of the gel. (C) Schematic of the transcript units analyzed in A and B. ATP9 and VAR1, tRNA16s, and CYTB as well as COX1 and ATP6/8 are cotranscribed.
Overexpression of RMD9 increases the steady-state level of mitochondrial mRNAs: Since our previous results suggested a post-transcriptional role for Rmd9p, we tested if the overexpression of Rmd9p could affect the steady-state levels of mitochondrial mRNAs encoding respiratory complex subunits, notably in the oxa1-E65G-F229S mutant.

The oxa1-E65G-F229S mutant and the wild-type strain were transformed with either the high-copy plasmid carrying RMD9 or the control empty vector. Total RNAs were extracted from the transformants grown at 28° and 36° and analyzed by hybridization with the RMD9, COX2, ATP9, 15S, and 21S probes and the ACT1 (actin) probe as a control. Similar results were obtained at both temperatures (Figure 6A; data not shown). The RMD9 mRNA was hardly detectable in the wild-type strain while it clearly accumulated when overexpressed. Overexpression of RMD9 in the oxa1 mutant also led to an increase in the RMD9 mRNA but to a level that was approximately half the level in the wild-type cells. Interestingly, overexpressing RMD9 in wild-type cells as well as in the oxa1 mutant led to an increase of both ATP9 and COX2 mRNAs—~3- to 4-fold for the ATP9 mRNA in both genetic backgrounds—whereas, for the COX2 mRNA, the effect was more pronounced in wild type—~6- to 7-fold compared to the oxa1 mutant at ~2-fold (Figure 6B). Finally, overexpression of RMD9 led to a moderate increase of ~2- and 1.5-fold of the 15S and 21S rRNAs, respectively, in both genetic backgrounds. The lower increases of RMD9 and COX2 mRNA levels in the oxa1 mutant compared to wild type could be explained by the difference in the respiratory capacity and ATP production of the cells. In particular, the difference in COX2 mRNA increase between wild-type and mutant cells upon RMD9 overexpression could be due to the strong sensitivity to ATP of the COX2 promoter (Amiot et al. 2006).

Our results show that overexpressing RMD9 leads to an increase in the steady-state level of several mitochondrial mRNAs in wild-type cells as well as in the oxa1-E65G-F229S mutant.

**DISCUSSION**

In this work we have isolated RMD9 as a high-copy suppressor of the respiratory-deficient mutant oxa1-E65G-F229S. We found that Rmd9p is a mitochondrial extrinsic membrane protein probably involved in the processing and/or stability of mitochondrial mRNAs encoding respiratory complex subunits. A homology search with Rmd9p led to the identification of another S. cerevisiae protein, Ybr238p (45% amino acid identity), that is also associated with mitochondrial membranes. Rmd9p-like proteins were found in *Kluyveromyces lactis* (38% amino acid identity), *Debaryomyces hansenii*, and in the pathogen *Candida albicans* (both 28% amino acid identity). In the *Candida glabrata* genome, two homologous proteins (~45% identity with Rmd9p) that could correspond, respectively, to Rmd9p and Ybr238p were found. Finally, no Rmd9p-like protein was found in other complete genome sequences, in particular in *S. pombe*. Proteins controlling mitochondrial RNA stability or translational activation are known to diverge rapidly, which probably reflects the rapid evolution of the untranslated RNA regions interacting with these proteins. However, Costanzo et al. (2000) have shown that, while the sequences of translational activator proteins are highly diverged in budding yeasts, their mRNA-specific functions are conserved. Similarly, the function
of Rmd9p-like proteins could be conserved. According to the PROSITE database (Falquet et al. 2002), the central region of Rmd9p contains a pentatrio peptide repeat (PPR)-like structural motif, which is found specifically in organelar proteins that bind to organelar transcripts (Lurin et al. 2004). Such a PPR motif was also found in two yeast mitochondrial proteins, Pet309p and Aep3p, that both control stability and translation of single mRNA (Manthey and McEwen 1995; Ellis et al. 2004; Krause et al. 2004). Rmd9p would be a new kind of RNA-binding factor belonging to the PPR family.

The inactivation of RMD9 led to a respiratory deficiency and a partial mtDNA loss, converting cells to rho−. Although Rmd9p and Ybr238p are related, no respiratory defect or rho− production were detected in the Δybr238 mutant and we have not found the YBR238 gene in our screen for high-copy suppressors of the mutant oxa1-E65G-F2298. However, the mtDNA appeared totally unstable in the double-mutant Δrmd9Δybr238, suggesting a functional interaction between the two proteins. The mtDNA instability observed in the Δrmd9 and Δrmd9Δybr238 mutants could be due to the strong reduction in the steady-state levels of ATP6/8 and ATP9 mRNAs that encode subunits of the membrane F0 sector of ATP synthase. Defects in F0 assembly have indeed been reported to lead to mtDNA instability (Tzagoloff and Dieckmann 1990). mtDNA loss has also been observed in various mutants affecting RNA processing, stability, and translation (for a review, see Contamine and Picard 2000).

In the absence of Rmd9p, all mitochondrial mRNAs encoding respiratory complex subunits were strongly reduced or undetectable, and unidentified large transcripts were detected for polycistronic RNAs. Such abnormal transcripts have been observed in mutants defective in mitochondrial mRNA processing/stability (e.g., Dieckmann et al. 1982; Groudinsky et al. 1993; Wiesenberger et al. 1997; Dziembowski et al. 2003; Ellis et al. 2004), suggesting that Rmd9p could be required for the processing and/or stability of a specific class of mitochondrial mRNAs. Three classes of proteins are already known to control the processing and/or stability of mitochondrial mRNAs, with various specificities. First, several factors, such as Cbp1p, Cbt1p, Pet309p, Nca2/3p, and Aep3p, are mRNA specific. Cbp1p and Cbt1p are required for CYTB mRNA stability and translation and interact with cis-elements located in the 5′ untranslated region (Dieckmann et al. 1982; Chen et al. 1999; Islas-Osuna et al. 2002; Ellis et al. 2005). Pet309p controls the stability and translation of COXI mRNA, interacts with Nam1p, and is associated with Cbp1p in a high-molecular-weight complex (Manthey and McEwen 1995; Naithani et al. 2003; Krause et al. 2004). Nca2/Nca3p and Aep3p stabilize the ATP6/8 mRNA (Camougrand et al. 1995; Ellis et al. 2004). Nam1p stabilizes the ATP6/8 mRNA and intron-containing transcripts; it interacts with the RNA poly-

merase (Groudinsky et al. 1993; Rodeheffer et al. 2001). Second, three proteins, Pet127p and the two subunits of the degradosome (Dss1p and Suv3p), play an important role in RNA surveillance and are more general factors (Wiesenberger et al. 1997; Dziembowski et al. 2003; Rogowska et al. 2006). In the Δpet127, Δdss1, or Δsuv3 mutants, unprocessed precursors or aberrant mitochondrial RNAs accumulate while the steady-state levels of several mRNAs, such as VAR1 and CYTB, decrease. And finally, a protein complex, as yet unidentified, binds to a conserved dodecamer sequence located at the 3′-end of all the mitochondrial RNAs (Osinga et al. 1984; Min and Zassenhaus 1993). The absence of this protein complex might lead to a general decrease of all mitochondrial transcripts. Compared to these various factors, Rmd9p appears to be the only protein that coregulates the steady-state levels of all mitochondrial mRNAs encoding respiratory complex subunits.

Rmd9p is an extrinsic membrane protein facing the mitochondrial matrix compartment. Most mitochondrial RNA-processing/stability factors, translational activators, and ribosomes are membrane bound or located in the vicinity of the inner membrane (for review, Haffer and Fox 1992; Fox 1996; Rodei 1997; Green-Wills et al. 1998; Naithani et al. 2003; Krause-Buchholz et al. 2005), which probably reflects the fact that mtDNA encodes mainly membrane proteins. Ybr238p, which interacts genetically with Rmd9p, is also a membrane-bound mitochondrial protein; further analysis will be undertaken to determine if it is associated in a protein complex with Rmd9p.

The absence of Rmd9p led to a slight reduction of VAR1 mRNA level and to a partial degradation of 15S RNA. Both RNAs encode components of the small subunit of the ribosome, which plays an important role in translation initiation. This decrease could be a secondary consequence of the strong decrease of other mitochondrial mRNA accumulation or could reveal a direct interaction of Rmd9p with the small subunit of the ribosome. We have performed 35S-Met labeling of mitochondrial translation products in Δrmd9, the level of incorporation was very low, preventing any conclusion from being drawn. This is not surprising, considering the effect on 15S RNA and the results of Williams et al. (2007, this issue) on the interaction of Rmd9p with ribosomes. A coupling between RNA stability and translation processes has already been reported for other factors controlling RNA stability (e.g., Cbp1p, Nam1p, Pet309p, and Pet127p; see above). Although Var1p is soluble, it seems to be translated near the inner membrane as are the membrane respiratory subunits (Fiori et al. 2003). However, the nontranslated region of VAR1 mRNA fused to COX2 or COX3 ORFs apparently caused mislocalized synthesis and degradation of the two subunits (Sanchirico et al. 1998). Sanchirico et al. (1998) suggested that there could be distinct sites of translation and assembly for mitochondrial ribosomes and for
mitochondria have already been proposed (the membrane insertion delay. Couplings between amounts of these subunits, thus compensating for specific increase in the amount of mRNAs coding for an assembly defect. Overexpressing mutations probably result in an insertion delay leading to subunits via interactions between its matrix domain and the large subunit of ribosomes (Ja et al. 2003; Szrych et al. 2003; Ott et al. 2006). Thus, oxal1 mutations probably result in an insertion defect. Overexpressing RMD9, through a specific increase in the amount of mRNAs coding for respiratory complex subunits, could lead to increased amounts of these subunits, thus compensating for the membrane insertion delay. Couplings between translation and assembly within and to the vicinity of mitochondria have already been proposed (e.g., Perez-Martinez et al. 2003; Barriontos et al. 2004; Margeot et al. 2005). RMD9 overexpression appears to act specifically on mutations located in the intermembrane space domain of Oxa1p, suggesting that both the membrane and matrix domains of Oxa1p are required for the suppression. In particular, it is likely that the interaction between the matrix domain of Oxa1p and ribosomes is required for the suppression activity.

In conclusion, Rmd9p and Oxa1p appear to play important roles in the expression of all mitochondrial genes encoding respiratory complex subunits. We propose that Rmd9p could convey the mRNAs to sites specific for the translation of respiratory subunits, where Oxa1p would then ensure the cotranslational insertion of these subunits within the membrane. Further experiments will be developed to depict the precise nature of these interactions.

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