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Alteration of a Novel Dispensable Mitochondrial Ribosomal Small-Subunit Protein, Rsm28p, Allows Translation of Defective *COX2* mRNAs

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Mutations affecting the RNA sequence of the first 10 codons of the *Saccharomyces cerevisiae* mitochondrial gene *COX2* strongly reduce translation of the mRNA, which encodes the precursor of cytochrome *c* oxidase subunit II. A dominant chromosomal mutation that suppresses these defects is an internal in-frame deletion of 67 codons from the gene *YDR494w*. Wild-type *YDR494w* encodes a 361-residue polypeptide with no similarity to proteins of known function. The epitope-tagged product of this gene, now named *RSM28*, is both peripherally associated with the inner surface of the inner mitochondrial membrane and soluble in the matrix. Epitope-tagged Rsm28p from Triton X-100-solubilized mitochondria sedimented with the small subunit of mitochondrial ribosomes in a sucrose gradient containing 500 mM NH_4Cl. Complete deletion of *RSM28* caused only a modest decrease in growth on nonfermentable carbon sources in otherwise wild-type strains and enhanced the respiratory defect of the suppressible *cox2* mutations. The *rsm28* null mutation also reduced translation of an *ARG8*^{m} reporter sequence inserted at the *COX1*, *COX2*, and *COX3* mitochondrial loci. We tested the ability of *RSM28-1* to suppress a variety of *cox2* and *cox3* mutations and found that initiation codon mutations in both genes were suppressed. We conclude that Rsm28p is a dispensable small-subunit mitochondrial ribosomal protein previously undetected in systematic investigations of these ribosomes, with a positive role in translation of several mitochondrial mRNAs.

Virtually all *Saccharomyces cerevisiae* mitochondrial ribosomal proteins are coded by nuclear genes, synthesized in the cytoplasm, and imported into the organelle, where they are assembled with a single protein and two rRNAs encoded in the mitochondrial DNA (23). Proteomic investigations of yeast mitochondrial ribosomal proteins have revealed that they contain a substantially larger number of polypeptides than bacterial ribosomes (19, 53). Mammalian mitochondrial ribosomes also contain a large number of proteins, all of which are encoded in the nucleus (36, 37, 45, 59, 60). Only about half of the proteins identified in mitochondrial ribosomes are detectably homologous to bacterial ribosomal proteins. This suggests that the remaining proteins could carry out novel ribosomal functions in mitochondrial translation systems.

The critical role of mitochondrial translation systems in cellular metabolism is to translate a handful of hydrophobic polypeptides from mitochondrially encoded mRNAs. These proteins must be inserted into the inner mitochondrial membrane and assembled into respiratory chain complexes (1). In *S. cerevisiae*, one adaptation for this specialized role of the translation system appears to be membrane-bound mRNA-specific translational activators that recognize targets in mRNA 5′ untranslated leaders (5′-UTLs) (16). The translational activators appear to both limit (24, 58) and localize (13, 16, 33, 38, 44, 50, 52) translation of mitochondrial mRNAs. Genetic evidence suggests that mitochondrial ribosomal proteins of the small subunit interact both with translational activators (27, 28, 40) and with targets in the mitochondrial mRNA 5′-UTLs (25). Biochemical and genetic evidence suggests that interaction between mitochondrial ribosomes and the conserved inner membrane protein Oxa1p facilitates insertion of mitochondrially coded proteins into the membrane (34, 61). A large-subunit ribosomal protein homologous to bacterial L23 can be cross-linked to Oxa1p (34). All of the ribosomal proteins identified in these functional studies are essential for mitochondrial translation.

In addition to the translational activator target in its 5′-UTL, the *COX2* mRNA contains antagonistic sequence elements with the pre-Cox2p coding sequence that control its translation: a positively acting element in the RNA sequence immediately downstream of the initiation codon, and negative elements further downstream in the coding sequence (3, 66). The mechanisms by which these elements function remain unknown. However, mutations within the first 10 codons of *COX2* that reduce translation can be suppressed in *cis* by nearby mutations that weaken a predicted stem structure (3). They can also be suppressed in *trans* by overexpression of the *COX2* mRNA-specific activator protein Pet111p as well as a mitochondrial ribosomal large-subunit protein, MrpL36p (3), which contains an essential core domain homologous to bacterial L31 and additional nonessential domains that cause suppression when overexpressed (67). Finally, dominant nuclear suppressors of these *cox2* mutations were isolated (3).

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype[a]	Source or reference
DL2	*MAT***a** *lys2* [*rho*$^+$]	15
DUL1	*MAT*α *lys2 ura3*Δ [*rho*$^+$]	15
EHW292	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 rsm28*Δ::*URA3* [*rho*$^+$, *cox2(1–91)*::*ARG8*^{m}]	This study
EHW385	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 rsm28*Δ::*URA3* [*rho*$^+$, *cox2*Δ::*ARG8*^{m}]	This study
EHW424	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 rsm28*Δ::*URA3* [*rho*$^+$, *cox2-22*]	This study
EHW463	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox1*Δ::*ARG8*^{m}]	This study
EHW464	*MAT***a** *arg8*Δ::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 rsm28*Δ::*URA3* [*rho*$^+$, *cox1*Δ::*ARG8*^{m}]	This study
EHW465	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox3*Δ::*ARG8*^{m}]	This study
EHW466	*MAT***a** *arg8*Δ::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 rsm28*Δ::*URA3* [*rho*$^+$, *cox3*Δ::*ARG8*^{m}]	This study
HMD22	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2*Δ::*ARG8*^{m}]	4
LSF74	*MAT***a** *lys2 ura3*Δ [*rho*$^+$, *cox3-1*]	14
NAB86	*MAT*α *ade2-101 ade3-24 rsm28*Δ::*URA3 leu2-3,112 ura3-52* [*rho*$^+$]	This study
NAB93	*MAT***a** *ade2-101 ade3-24 arg8*::*hisG rsm28*Δ::*URA3 leu2-3 leu2-112 ura3-52* [*rho*$^+$, *cox2-22*]	This study
NAB97	*MAT***a** *arg8*::*hisG RSM28-HA his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-22*]	This study
NAB106	*MAT***a** *arg8*::*hisG RSM28-1-HA his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-22*]	This study
NAB108	*MAT*α *ade2-101 ade3-24 rsm28*Δ::*URA3 leu2-3,112 ura3-52* [*rho*$^+$, *cox2-27*]	This study
NAB109	*MAT***a** *arg8*Δ::*hisG rsm28*Δ::*URA3 his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-22*::*ARG8*^{m}]	This study
NAB111	*MAT***a** *arg8*::*hisG rsm28*Δ::*URA3 his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2*::*ARG8*^{m}]	This study
NAB112	*MAT***a** *arg8*::*hisG rsm28*Δ::*URA3 his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-27*::*ARG8*^{m}]	This study
NAB119	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2*::*ARG8*^{m}]	This study
NAB120	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-22*]	This study
NAB121	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-22*]	This study
NAB122	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-27*]	This study
NAB123	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-22*::*ARG8*^{m}]	This study
NAB124	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-27*::*ARG8*^{m}]	This study
NAB125	*MAT*α *ade2-101 ade3-24 leu2-3,112 ura3-52 pDB20* [*rho*$^+$]	This study
NAB126	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$]	This study
NAB127	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2*::*ARG8*^{m}]	This study
NAB128	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-22*::*ARG8*^{m}]	This study
NAB129	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-27*::*ARG8*^{m}]	This study
NAB130	*MAT***a** *arg8*::*hisG RSM28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52 pDB20* [*rho*$^+$, *cox2-27*]	This study
NB40-3C	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-62*]	4
NB43	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2(1-91)*::*ARG8*^{m}]	4
NB60	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-10*]	4
NB64	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-22*]	3
NB64-S7c	*MAT***a** *arg8*::*hisG RMS28-1 his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*+, *cox2-22*]	This study
NB80	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$]	4
NB110	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-10(1-91)*::*ARG8*^{m}]	4
NB134	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-34(1-91)*::*ARG8*^{m}]	4
NB164	*MAT***a** *arg8*::*hisG his3*Δ*HindIII leu2-3,112 lys2 ura3-52* [*rho*$^+$, *cox2-34*]	4

[a] Mitochondrial genotypes are in brackets.

In this study we describe an as-yet-undetected yeast mitochondrial ribosomal small-subunit protein, Rsm28p, as the product of a gene identified by one of the previously isolated dominant nuclear suppressor mutations. Interestingly, the suppressor mutation is an internal in-frame deletion that alters the structure and function of Rsm28p. Furthermore, Rsm28p is not essential for mitochondrial translation, unlike other mitochondrial ribosomal proteins identified genetically by functional screens (25, 27, 28, 40).

MATERIALS AND METHODS

Strains, media, and genetic techniques. The *S. cerevisiae* strains used in this study are listed in Table 1. They are isogenic or congenic to strain D273-10B (ATCC 25627). Fermentable complete medium was YPAD or YPAGal containing 1% yeast extract, 2% Bacto-Peptone, 100 mg of adenine/liter, and either 2% glucose or 2% galactose supplemented with 0.1% glucose. Nonfermentable medium was YPEG containing 1% yeast extract, 2% Bacto-Peptone, 100 mg of adenine/liter, 3% ethanol, and 3% glycerol. Minimal medium (0.67% yeast nitrogen base without amino acids) was supplemented with specific amino acids, uracil, and adenine as required, and either 2% glucose or 3% ethanol and 3% glycerol for nonfermentable medium. Standard genetic methods were as previously described (17, 26, 56). *S. cerevisiae* nuclear transformation was carried out with the Frozen-EZ Yeast Transformation II kit (Zymo Research) or lithium acetate (9).

Cloning of the suppressor gene. A library of DNA from the suppressor strain NB64-S7c was prepared by partially digesting genomic DNA with Sau3AI. Restriction fragments were fractionated on sucrose gradients (10 to 40%) (51), and the 10-kb population was cloned into the BclI-cut vector pNB34. pNB34 is a low-copy-number shuttle plasmid with *URA3* and ampicillin resistance selections in *S. cerevisiae* and *Escherichia coli*, respectively. Inserts are cloned into the λ *cI* gene, which regulates expression of a kanamycin resistance cassette, allowing selection of insert-containing plasmids (30). The resulting library was used to transform NB64. Transformants (≈7,500) were screened for suppression of the respiration defect at 30 and 16°C. Respiring colonies that appeared at both temperatures were isolated and tested for linkage of respiration to the plasmid.

Plasmids were isolated from four strains, and their suppression phenotype was confirmed by transformation of NB64. Two plasmids were sequenced from both ends, revealing a stretch of DNA sequence (≈14 kb) from chromosome IV with 11 possible open reading frames (ORFs). To determine which of these ORFs was responsible for the suppression phenotype, the GPS-1 Genome Priming System (New England Biolabs, Inc., Beverly, Mass.) for in vitro Tn*7* mutagenesis was used. The Tn*7*-mutagenized plasmid library was transformed into NB64 and screened for the loss of suppression of respiration. Five plasmids were isolated and sequenced from both arms of Tn*7*, revealing insertion of Tn*7* in all plasmids into different positions in the same ORF, *YDR494w*, which we have named *RSM28*.

***YDR494w*/*RSM28* sequence correction, disruption, and hemagglutinin tagging.** *YDR494w*/*RSM28* was PCR amplified from both NB64 and NB64-S7c to determine the nature of the suppressor mutation. The sequences of the PCR products lack one copy of a 16-base tandem repeat (TGATCCTCCTTGATTT) reported in the original published sequence for wild-type *YDR494w* (22), extending the protein from a putative size of 288 to 361 amino acids. We confirmed this sequence correction in DNA from both FY23 and strains in the D273-10B (ATCC 25657) background used here (GenBank accession no. AF459095). This has been corrected in the Saccharomyces Genome Database reference sequence. The suppressor allele, termed *RSM28-1* (GenBank accession no. AF459096), has an internal in-frame deletion of 67 amino acids (amino acids 120 to 186), which are flanked by a GCAGC direct repeat.

For functional testing, genomic fragments carrying *RSM28* and *RSM28-1* were amplified by PCR from genomic DNA, cut with DraI, and subcloned into the SmaI site of the multicopy 2μm vector YEp352 (32) to yield pEHW217 (*RSM28*) and pEHW218 (*RSM28-1*). The clones were sequenced to confirm the absence of PCR-induced mutations. The *RSM28* and *RSM28-1* fragments from pEHW217 and pEHW218 then were subcloned as SacI-XbaI fragments into the same sites of the *CEN* vector pRS316 (57) to yield plasmids pEHW219 (*RSM28*) and pEHW220 (*RSM28-1*), respectively.

RSM28 was disrupted by replacement of nucleotides −16 to +854 (with respect to the translation initiation codon) with a PCR-derived *URA3* cassette introduced into the genome by homologous recombination and checked by PCR. To tag the products of *RSM28* and *RSM28*-1 with three hemagglutinin (HA) epitopes, an *HA-URA3-HA* cassette (54) flanked by *RSM28* C-terminal coding sequence was amplified by PCR and transformed into NB64 and NB64-S7c. Due to the design of the tagging cassette, the tagged proteins bear 12 additional amino acids after the epitope. Following pop-out of the *URA3* marker, the tag junction and coding sequence were confirmed by PCR and sequencing.

Northern and Western analyses. RNA isolation and Northern blotting procedures have been described previously (66). Hybridization probes were, for *COX2*, a 1.6-kb PacI fragment from plasmid pJM2 (42) and, for 15S rRNA, XhoI-linearized plasmid pT82 (55). Total cellular protein extracts were prepared as described previously (68). Proteins of interest were visualized by electrophoresis of 50 to 100 μg of total protein per sample on sodium dodecyl sulfate–12% acrylamide gels, followed by Western blotting. The following antisera were used: mouse monoclonal anti-Cox2p (47); rabbit polyclonal anti-Arg8p (58); rabbit polyclonal anti-glucose-6-phosphate dehydrogenase (G6PDH) (Sigma-Aldrich); mouse monoclonal anti-Mrp7p (11); mouse monoclonal anti-Mrp13p (46); rabbit polyclonal anti-Hsp60p (29); rabbit polyclonal anti-Yme1p (62); rabbit polyclonal anti-citrate synthase, rabbit polyclonal anti-cytochrome b_2, and horseradish peroxidase-conjugated mouse monoclonal anti-HA (Roche Molecular Biochemicals). For non-horseradish peroxidase-conjugated mouse antisera, secondary detection utilized 1:5,000 to 1:10,000 (Bio-Rad Laboratories) or 1:10,000 (Sigma-Aldrich) horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G; for rabbit antisera, 1:10,000 to 1:20,000 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Invitrogen or Bio-Rad Laboratories). Chemiluminescent signal was visualized with the ECL or ECL Plus detection kit (Amersham Pharmacia Biotech).

Mitochondrial isolation, subfractionation, and protein analyses. Mitochondrial isolation and purification, mitochondrial membrane fractionation, and alkaline extraction, mitoplasting, proteinase K protection assay, in vivo pulse-labeling and Western blot analysis were as previously described (3, 18, 20, 21, 31). Mitochondrial ribosomes were extracted from purified mitochondria (1 mg of protein) resuspended in 1 ml of 10 mM magnesium acetate–0.1 M NaCl–20 mM HEPES-KOH (pH 7.4)–1 mM phenylmethylsulfonyl fluoride by the addition of Triton X-100 to 0.5% and incubation on ice for 30 min. The lysate was clarified by centrifugation at 34,000 rpm for 20 min at 4°C in a Beckman TLA100.3 rotor (40,000 × *g*) and layered onto a 39-ml continuous 15 to 30% sucrose gradient containing 500 mM NH_4Cl, 10 mM Tris, 10 mM magnesium acetate, pH 7.4, 7 mM beta-mercaptoethanol, 0.2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and one complete protease inhibitor minitablet without EDTA (Roche). Gradients were centrifuged at 20,000 rpm for 17 h at 4°C in a Beckman SW28 rotor, and 1-ml fractions were collected; 0.4 ml of each fraction analyzed was concentrated by trichloroacetic acid precipitation and subjected to SDS gel electrophoresis and Western blotting.

RESULTS

A dominant nuclear mutation suppresses mitochondrial mutations that alter the pre-Cox2p leader peptide coding sequence and reduce *COX2* mRNA translation. Two mutations, *cox2-22* and *cox2-27*, alter the pre-Cox2p leader peptide coding region of the mitochondrially coded *COX2* mRNA and reduce its translation (3). *cox2-22* is a compound allele with a deletion of codons 7 to 10 and the translationally silent change of codon 6 from AGA to CGT (3). *cox2-27* is a deletion of codons 2 to 6 (3). We previously isolated spontaneous pseudorevertants of the *cox2-22* mutant strain NB64 with improved respiratory growth and found that roughly half contained dominant nuclear suppressor mutations that were not characterized (3).

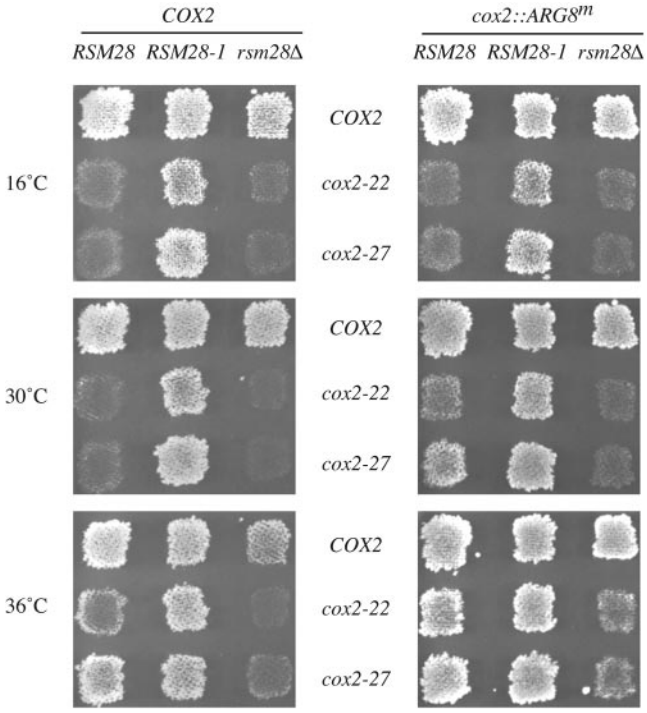

FIG. 1. *RSM28* alleles affect respiratory growth and mitochondrial reporter activity in *cox2-22* and *cox2-27* strains. Strains with the indicated nuclear wild-type *RSM28*, suppressor *RSM28-1*, or deletion *rsm28*Δ::*URA3* alleles and the indicated mitochondrial alleles in an intact *COX2* locus or the *cox2*::*ARG8*^{m} reporter fusion were grown as patches on minimal glucose medium and then replica plated to nonfermentable minimal medium for the *COX2* alleles (left panels) or minimal glucose medium lacking arginine for the *cox2*::*ARG8*^{m} alleles (right panels). Plates were incubated at 16, 30, or 36°C as indicated and were photographed after 2 to 6 days. Strains used (from left to right): *COX2*; NAB125, NAB126, and NAB86; *cox2-22*; NAB120, NAB121, and NAB93; *cox2-27*; NAB122, NAB130, and NAB108; *cox2*::*ARG8*^{m}; NAB119, NAB127, and NAB111; *cox2-22*::*ARG8*^{m}; NAB123, NAB128, and NAB109; and *cox2-27*::*ARG8*^{m}; NAB124, NAB129, and NAB112. As indicated in Table 1, strains lacking a chromosomal *URA3* gene were transformed with the *URA3* plasmid pDB20 (12).

In this study, we focused on the revertant containing the strongest of these nuclear suppressors, NB64-S7c. Its suppressor mutation, termed *RSM28-1* for reasons described below, improved respiratory growth on nonfermentable carbon sources of strains bearing either the *cox2-22* or *cox2-27* mutation (Fig. 1), and greatly increased the steady-state level of Cox2p in strains bearing the *cox2-22* mutation (Fig. 2).

To ask whether the suppressor affects the level of gene expression from this locus, as opposed to a downstream step in cytochrome oxidase assembly, we tested its effect on strains bearing the *cox2-22* and *cox2-27* mitochondrial mutations in a

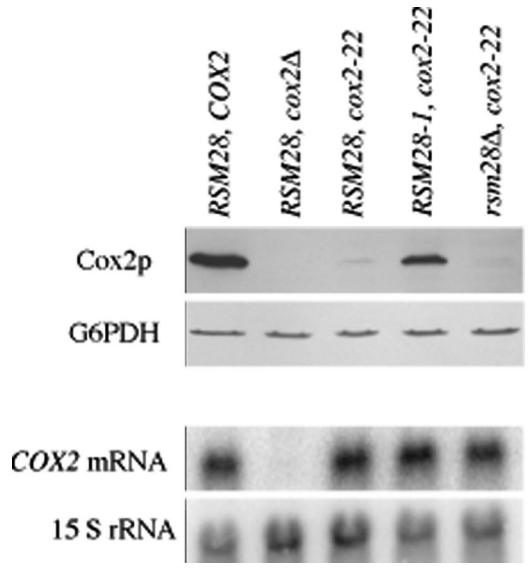

FIG. 2. *RSM28-1* increases Cox2p accumulation in a *cox2-22* mutant without affecting *COX2* mRNA levels. Strains containing wild-type *RSM28*, suppressor *RSM28-1*, or deletion *rsm28*Δ::*URA3* genomic alleles and wild-type *COX2*, a *cox2* deletion, or *cox2-22* mitochondrial alleles were grown to mid-log phase in complete medium containing galactose (YPAGal), and protein and RNA were extracted (from separate cultures). The top two panels show a Western blot of 100 μg of total cellular protein probed with anti-Cox2p antibody (top panel), and anti-glucose-6-phosphate dehydrogenase (G6PDH) as a loading control (second panel). The bottom two panels show a Northern blot of 10 μg of total cellular RNA probed with a genomic fragment containing *COX2* (third panel) and a probe for 15S rRNA as a loading control (bottom panel). Relevant genotypes are indicated above the lanes. Strains are described in Table 1: *RSM28 COX2* (NB80); *RSM28 cox2*Δ (NB40-3C); *RSM28 cox2-22* (NB64); *RSM28-1 cox2-22* (NB64-S7c); and *rsm28*Δ *cox2-22* (EHW424).

cox2::*ARG8*^{m} reporter gene (3). This reporter expresses the arginine biosynthetic enzyme Arg8p from the *COX2* locus as a fusion to the 91st residue of pre-Cox2p and reliably reports *COX2* mRNA translation (3, 4, 66). The *RSM28-1* suppressor improved the growth of *cox2-22*::*ARG8*^{m} and *cox2-27*::*ARG8*^{m} strains on glucose medium lacking arginine (Fig. 1), indicating that it increased gene expression per se. The *cox2-22* mutation has no significant effect on the steady-state level of *COX2* mRNA relative to that of wild-type (3), and the presence of the suppressor mutation *RSM28-1* did not increase the level of *cox2-22* mRNA dramatically, if at all (Fig. 2). Thus, *RSM28-1* appears to improve translation of the mutant mRNAs.

Identification of the suppressor mutation, *RSM28-1*, as an in-frame deletion within *YDR494w*. To identify the suppressor, we first tested for genetic linkage of the suppressor mutation to the candidate genes *PET111*, *MRP21*, and *MRP51* (25, 41), but no linkage was observed. We therefore constructed a genomic library of DNA isolated from the suppressed mutant strain NB64-S7c in the *CEN* vector pNB34 (Materials and Methods) and screened it for plasmids that could suppress the Pet$^-$ phenotype of the *cox2-22* mutant strain NB64. One suppressing plasmid with 11 putative genes from chromosome IV was obtained. To identify the gene responsible for suppression, this plasmid was mutagenized in vitro with the transposon Tn*7* (Materials and Methods). Five mutagenized plasmids that had lost the ability to suppress *cox2-22* were isolated and sequenced, revealing distinct Tn*7* insertions into ORF *YDR494w*, now termed *RSM28*. *RSM28*/*YDR494w* is a unique gene predicted to encode a 361-residue protein that exhibits no significant sequence similarity to any other known or predicted protein other than its homologues in closely related fungi (5, 6, 35). (The *YDR494w* ORF in the original reference genomic sequence of strain S288c [22] predicted a 288-amino-acid protein; see Materials and Methods.)

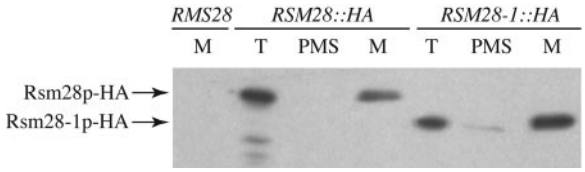

FIG. 3. Rsm28p cofractionates with mitochondria. Cells were grown in YPAGal at 30°C to late log phase, disrupted to yield total cell extracts (T), and fractionated into crude mitochondrial pellets and postmitochondrial supernatants (PMS), and crude mitochondria were then purified on Nycodenz gradients (M). Extracts from untagged wild-type *RSM28* (DL2), *RSM28*::*HA* (NAB97), and *RSM28-1*::*HA* (NAB106) strains were resolved on sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, immunoblotted, and probed with anti-HA monoclonal antibody. Arrows indicate immunoreactive bands representing epitope-tagged Rsm28p-HA and the suppressor Rsm28-1p-HA.

DNA sequence analysis of *RSM28-1*, the dominant suppressor mutation, revealed an internal in-frame deletion of 67 codons specifying amino acids 120 to 186 (GenBank accession no. AF459096). This result confirmed that *YDR494w* corresponds to the suppressor gene *RSM28* and indicates that the shortened protein, Rsm28-1p, apparently acts to suppress the mitochondrial *cox2-22* and *cox2-27* mutations.

Rsm28p is a small-subunit mitochondrial ribosomal protein. To examine wild-type Rsm28p and the suppressor form of the protein, we tagged the C termini of both by inserting DNA encoding three HA epitopes at the ends of the chromosomal ORFs (Materials and Methods). The epitopes did not abolish Rsm28p function, since expression of the HA-tagged suppressor protein Rsm28-1p-HA suppressed the *cox2-22* mutation. Expression of the wild-type tagged protein Rsm28p-HA did not diminish the leaky growth phenotype caused by *cox2-22*, in contrast to the *rsm28* null mutation. Indeed, the Rsm28p-HA protein caused slightly improved respiratory growth of *cox2-22* strains compared to strains expressing the unmodified Rsm28p (unpublished data). Thus, both tagged proteins appear to be functional.

A total extract of cells expressing wild-type Rsm28p-HA contained an immunoreactive protein of the appropriate size (Fig. 3). As expected, the corresponding immunoreactive protein in a total extract of cells expressing the suppressor protein, Rsm28-1p-HA, was shorter due to the internal in-frame deletion (Fig. 3). Highly purified mitochondria prepared from both the wild-type and suppressor strains bearing the HA tag were enriched for these proteins, while the postmitochondrial supernatants contained very little (Fig. 3). Purified mitochondria from a wild-type strain lacking any HA tag did not contain this immunoreactive species (Fig. 3).

To determine the submitochondrial location of Rsm28p-

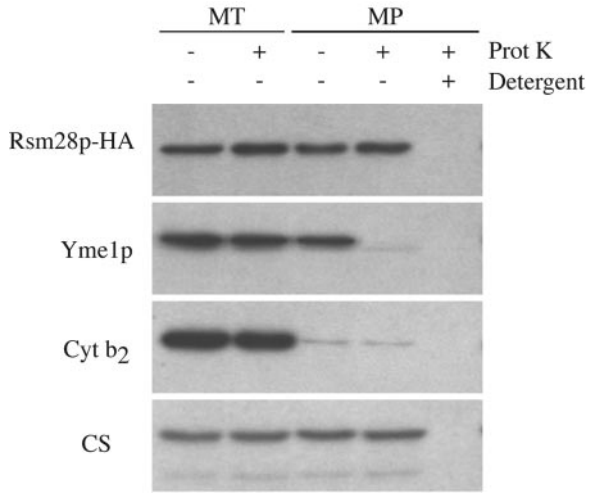

FIG. 4. Rsm28p is within the inner membrane of mitoplasts. Mitochondria (MT) were purified from strain NAB97 (*RSM28*::*HA*) and converted to mitoplasts (MP) by osmotic shock in the absence or presence of 20 μg of proteinase K (Prot K) per ml, as indicated. Mitoplasts also were treated with proteinase K in the presence of 1% octylglucoside (detergent) to solubilize the inner membrane. Treated samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotted, and probed with anti-HA (Rsm28p-HA), anti-Yme1p as an integral inner membrane protein marker (Yme1p), anti-cytochrome b_2 as an intermembrane space marker (Cyt b_2), and anti-citrate synthase as a matrix marker (CS).

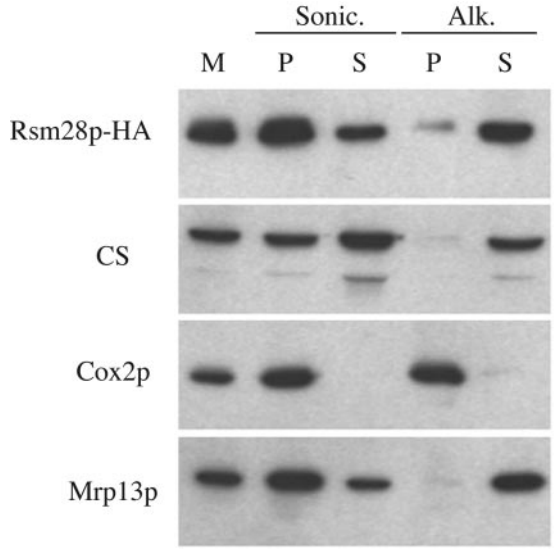

FIG. 5. Rsm28p is peripherally associated with mitochondrial membranes. Mitochondria (M) purified from a strain expressing Rsm28p-HA (NAB97) were sonicated (Sonic.) and separated into a membrane pellet (P) and soluble supernatant (S) by centrifugation. An aliquot of this membrane fraction was extracted with sodium carbonate (pH 11.5) (Alk.), followed by centrifugation to separate pelleted integral membrane proteins (P) from solubilized proteins in the supernatant (S). Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotted, and probed with anti-HA antibody (Rsm28p-HA), anti-citrate synthase as a matrix marker (CS), anti-Cox2p as an integral inner membrane marker (Cox2p), and anti-Mrp13p (Mrp13p) as a mitochondrial ribosomal marker.

HA, we first examined its sensitivity to digestion by exogenous protease added to mitochondria and to mitoplasts lacking the outer membrane. Rsm28p-HA was resistant to protease in intact mitochondria and in mitoplasts, while detergent solubilization of mitoplasts rendered Rsm28p-HA sensitive to protease (Fig. 4). This behavior was similar to that of the matrix marker protein citrate synthase (Fig. 4). In contrast, Yme1p, an inner membrane protein exposed on the outer surface, was susceptible to protease degradation in mitoplasts (Fig. 4). Thus, Rsm28p-HA appears to reside inside the inner membrane.

We next asked whether Rsm28p-HA is soluble or membrane bound by sonicating purified mitochondria and separating membrane and soluble fractions by centrifugation. Rsm28p-HA was detected primarily in the membrane pellet but also in the soluble supernatant (Fig. 5). Extraction of the pelleted membranes with alkaline sodium carbonate (18) solubilized almost all the Rsm28p-HA, indicating that it is peripherally associated with membranes. We obtained similar results with the tagged suppressor protein Rsm28-1p-HA (not shown). The behavior of Rsm28p-HA in these experiments closely paralleled that of Mrp13p (Fig. 5), a known mitochondrial small ribosomal subunit protein (46).

To test whether Rsm28p-HA is a component of mitochondrial ribosomes, purified mitochondria containing Rsm28p-HA were solubilized with Triton X-100, and the resulting lysate was sedimented in the presence of the detergent into a sucrose gradient containing 0.5 M NH_4Cl (Materials and Methods). The gradient fractions were analyzed by probing Western blots with anti-HA and antisera against control proteins (Fig. 6). The sedimentation behavior of Rsm28p-HA was essentially identical to that of the mitochondrial ribosomal small-subunit protein Mrp13p, sedimenting ahead of the Hsp60 complex (48) and behind the mitochondrial ribosomal large-subunit protein Mrp7p (11). (A significant proportion of Mrp7p and an apparent degradation product remained near the top of these gradients.) Similar results were obtained when Rsm28p and Rsm28-1p were labeled with a twin affinity purification (49) tag (unpublished results).

Taken together with the submitochondrial localization of Rsm28p-HA, these data strongly indicate that Rsm28p is a small-subunit mitochondrial ribosomal protein. Consistent

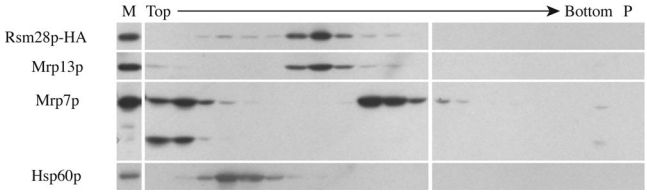

FIG. 6. Rsm28p cosediments with the mitochondrial ribosomal small subunit. Mitochondria were isolated from a strain expressing Rsm28p-HA (NAB97). Mitochondrial ribosomes were extracted by treatment with Triton X-100 and sedimented through continuous 15 to 30% sucrose gradients containing 0.5 M NH_4Cl. Alternate fractions of the gradient were precipitated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotted, and probed with anti-HA antibody (Rsm28p-HA), anti-Mrp13p (Mrp13p) as a small-subunit standard, anti-Mrp7p (Mrp7p) as a large-subunit standard, and anti-Hsp60p (Hsp60p). M, total mitochondria; P, solubilized sucrose gradient pellet.

with this identification, the level of Rsm28p-HA in ρ^0 cells, which cannot assemble ribosomal subunits, was dramatically lower than in ρ^+ cells (unpublished results) as has been previously observed for the small-subunit proteins Mpr13p (46) and Pet123p (40). Following the systematic nomenclature suggestion of Saveanu et al. (53) for small-subunit mitochondrial ribosomal proteins, we have renamed *YDR494w* as *RSM28*.

Rsm28p is required for normal translation but is not essential. We deleted the first 285 codons (out of 361) of the chromosomal copy of *RSM28* by replacing them with *URA3* (Materials and Methods). This null mutation, *rsm28*Δ::*URA3*, had little effect on the respiratory growth of an otherwise wild-type strain. The only phenotypes observed were a modest reduction in respiratory growth at 36°C (Fig. 1) and a modest increase in sensitivity to H_2O_2 in mutant cells growing on nonfermentable carbon sources (unpublished results). However, when combined with the *cox2-22* and *cox2-27* mutations or with the *cox2-22*::*ARG8*^{m} and *cox2-27*::*ARG8*^{m} reporters, *rsm28*Δ::*URA3* reduced the residual growth caused by these leaky mitochondrial mutations (Fig. 1). As expected, the null allele was recessive to both wild-type *RSM28* and the suppressor allele *RSM28-1* in heterozygous diploid strains (unpublished data). Tetrad analysis of spores from a diploid heterozygous for *rsm28*Δ::*URA3* and *RSM28-1* confirm the linkage of the suppressor to this locus (unpublished data).

The fact that deletion of the gene coding ribosomal protein Rsm28p does not completely block mitochondrial translation is surprising but not unprecedented (10, 19). Despite the subtle respiratory growth defect detectable in *rsm28*Δ::*URA3 COX2* strains on nonfermentable carbon sources (Fig. 1), no significant decrease in steady-state Cox2p accumulation is detectable in stationary-phase cells grown on galactose (unpublished data). However, we have previously observed that steady-state levels of the Arg8p reporter are more sensitive indicators of translational defects than levels of Cox2p (4).

We therefore examined the effect of *rsm28*Δ::*URA3* on the expression of two different *cox2*::*ARG8*^{m} reporters: one has *ARG8*^{m} fused to the 91st *COX2* codon, as described above, while the other has no *COX2* codons. In both cases, the absence of Rsm28p substantially reduced the level of accumulated Arg8p, indicating a significant reduction in translation of the reporter mRNAs (Fig. 7). (The Arg8p accumulating in these strains is nevertheless sufficient to support normal Arg^+ growth [Fig. 1].) Furthermore, expression of the *ARG8*^{m} reporter inserted in place of the *COX1* and *COX3* coding sequences was similarly reduced in the absence of Rsm28p (Fig. 7)

Allele specificity of suppression by plasmid-borne *RSM28-1*. To test the ability of *RSM28-1* to suppress other mitochondrial mutations, we first established that it could suppress *cox2-22* when borne on a plasmid. Genomic fragments containing *RSM28-1* or *RSM28* with the endogenous promoter were inserted into centromeric and 2μm plasmids (Materials and Methods) and transformed into a *cox2-22* mutant strain. The presence of *RSM28-1* on a centromeric plasmid suppressed *cox2-22*, as would be expected for the dominant suppressor allele (Fig. 8A). Additional copies of *RSM28-1* carried on the 2μm plasmid suppressed more strongly (Fig. 8A). Wild-type *RSM28* failed to suppress *cox2-22* when carried on either the

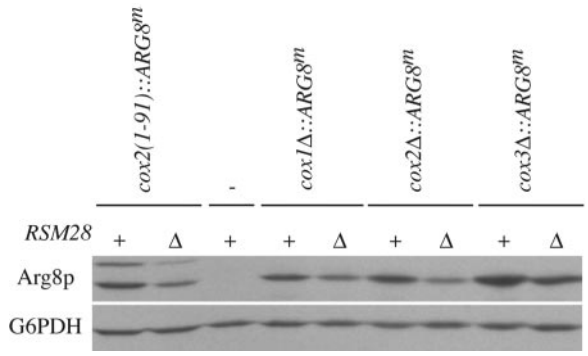

FIG. 7. Rsm28p is required for full expression of the *ARG8*^{m} reporter inserted at the *COX1*, *COX2*, and *COX3* loci. Western blots of 50 μg of total cellular protein extracted from cells grown to mid- to late log phase in complete medium containing galactose (YPAGal) were probed with anti-Arg8p. The relevant mitochondrial genotypes of each strain are shown above the horizontal lines: − indicates wild-type mitochondrial DNA lacking *ARG8*^{m}. The relevant nuclear genotypes are wild-type *RSM28* (+) and the null allele *rsm28*Δ::*URA3* (Δ). All strains lack the nuclear *ARG8* gene. The blot was reprobed with anti-glucose-6-phosphate dehydrogenase (G6PDH) as a loading control. Strains utilized (from left to right): NB43, EHW292, NB80, EHW463, EHW464, HMD22, EHW385, EHW465, and EHW466.

centromeric or 2μm plasmid (Fig. 8A), although this gene did complement the *rsm28*Δ::*URA3* null allele (unpublished data).

To investigate the allele specificity of suppression by *RSM28-1*, we transformed the high-copy-number 2μm plasmid bearing *RSM28-1* into a diverse collection of strains with mitochondrial mutations in *COX2* and *COX3*. We observed no suppression of any mutation tested affecting the mRNA 5′-UTL sequences (8, 42, 65) or of any missense or nonsense mutations (unpublished results). Several of these mutations have leaky phenotypes, making them sensitive indicators for a general increase in gene expression. Therefore, the fact that they were not suppressed suggests that *RSM28-1* does not globally increase translation.

Interestingly, *RSM28-1* did suppress both *cox2* and *cox3* initiation codon mutations. AUA in place of AUG at either gene reduces but does not eliminate translation (15, 43). AUU in place of AUG at *COX2* allows more efficient translation than AUA (4). *RSM28-1* on the 2μm plasmid clearly improved the respiratory growth of *cox2* and *cox3* mutants with AUA initiation codons as well as the *cox2* mutant with AUU (Fig. 8B). This suppression was also evident when scored by expression of the *cox2*::*ARG8*^{m} reporter containing *COX2* codons 1 to 91 and the AUG to AUU mutation (Fig. 8B). Almost no suppression was observed with AUA in the *cox2*::*ARG8*^{m} reporter, consistent with previous results showing that this is a tighter mutation (4). We conclude that the suppressor activity of Rsm28-1p appears to be limited to lesions affecting the coding sequence specifying the extreme N-terminal regions of mitochondrial proteins, but it is clearly not gene specific.

DISCUSSION

We have identified a novel yeast mitochondrial ribosomal small-subunit protein, Rsm28p, that had previously gone undetected in systematic proteomic analyses of these ribosomes (19, 53). Interestingly, Rsm28p is partially dispensable for mi-

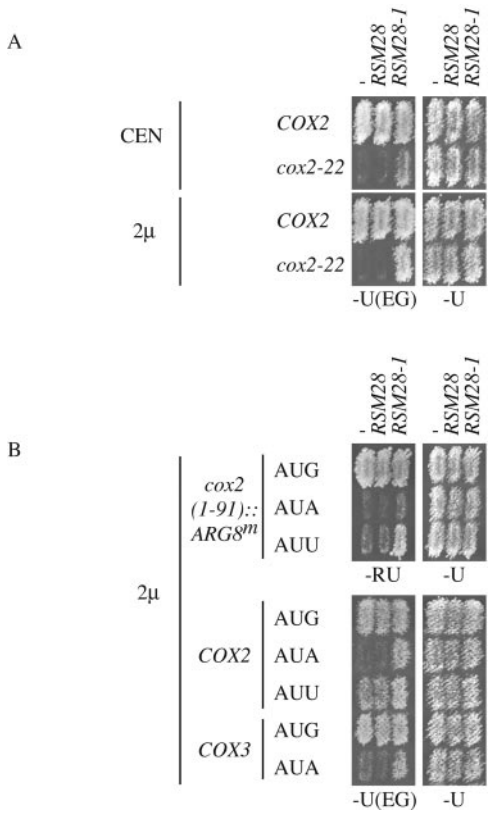

FIG. 8. Plasmid-borne *RSM28-1* can suppresses *cox2-22*, as well as *cox2* and *cox3* initiation codon mutations. (A) *COX2* (NB80) and *cox2-22* (NB64) strains were transformed with centromeric (*CEN*) or 2μm multicopy plasmids carrying *RSM28* or *RSM28-1*, as indicated. − indicates empty vector. Transformants were replica plated to nonfermentable medium [−U(EG)] and glucose medium (−U), selecting for the plasmid marker, and incubated for 4 and 2 days at 30°C, respectively. The *CEN/ARS* plasmids indicated as −, *RSM28*, and *RSM28-1* were pRS316, pEHW219, and pEHW220, respectively (Materials and Methods). The 2μm plasmids indicated as −, *RSM28*, and *RSM28-1* were YEp352, pEHW217, and pEHW218, respectively (Materials and Methods). (B) Wild-type (AUG) and initiation codon mutant (AUA and AUU) strains were transformed with 2μm plasmids expressing *RSM28* or *RSM28-1*. *cox2(1–91)*::*ARG8*^{m} strains (top panels) were replica plated to medium selecting for Arg^+ growth (−RU) and medium selecting for the plasmids (−U). *COX2* and *COX3* strains (bottom panels) were replica plated to nonfermentable medium [−U(EG)] and glucose medium, selecting for the plasmid marker (−U). Incubation was for 2 days at 30°C for −U and −U(EG) and 4 days for −RU. Strains used (from top to bottom): NB43, NB110, NB134, NB80, NB60, NB164, DUL1, and LSF74. The 2μm plasmids were as in A.

tochondrial gene expression, although deletion of its gene causes reduced respiratory growth, particularly at elevated temperature, and decreases the expression of a reporter gene inserted into mitochondrial DNA at the *COX1*, *COX2*, and *COX3* loci. Thus, Rsm28p appears to play a role in normal translation of at least several mitochondrially coded mRNAs. While nonessential ribosomal proteins are unusual, they have previously been described in both cytoplasmic (2, 7) and mitochondrial (10) ribosomes of *S. cerevisiae*.

We were led to the gene *RSM28* (*YDR494w*) by its functional interaction with the mitochondrially encoded *COX2* mRNA. A spontaneous in-frame deletion mutation, *RSM28-1*, was selected as a dominant suppressor of *COX2* mRNA translation defects caused by *cox2* mutations that altered the sequence immediately downstream of the *COX2* initiation codon (3). This suppression is not simply due to loss of *RSM28* function since the complete *rsm28* deletion mutation fails to suppress. Indeed, the complete absence of Rsm28p reduces translation of wild-type and mutant *COX2* mRNAs as well as *cox1*Δ::*ARG8*^{m} and *cox3*Δ::*ARG8*^{m} reporter mRNAs. Thus, wild-type Rsm28p enhances the ability of ribosomes to initiate and/or elongate polypeptide synthesis, and the shorter suppressor form, Rsm28-1p, appears to have increased or altered this activity. Interestingly, we found that the *RSM28-1* dominant suppressor mutation also weakly suppresses leaky *cox2* and *cox3* initiation codon mutations. This finding suggests the possibility that Rsm28p could play a general role in translation initiation. In this connection, it is interesting that two proteins with known roles in mitochondrial translation initiation, initiation factor 2, Ifm1p (63, 64), and methionyl-tRNA formyltransferase, Fmt1p (39), are also partially dispensable for yeast mitochondrial translation.

Translation of the *COX2* mRNA appears to be controlled in a complex fashion. The *COX2* mRNA-specific translational activator Pet111p works through a site in the mRNA 5′-UTL to promote synthesis of downstream coding sequences (4, 8, 41, 42) by an unknown mechanism. Genetic interactions also implicate two small-subunit ribosomal proteins, Mrp21p (a homolog of bacterial S21) and Mrp51p, in recognition of the *COX2* mRNA 5′-UTL, although their activity is not mRNA specific and they are essential for global mitochondrial translation (25). Thus, it appears that translational activators and the ribosomes work together to recognize mRNA 5′-UTLs and probably initiation codons.

Additional antagonistic elements controlling *COX2* mRNA translation are present within the coding sequence itself. A positive element is embedded in the mRNA sequence of the first 14 codons, which specify the leader peptide of pre-Cox2p (3), while a negative element is embedded in the mRNA sequence of codons 15 to 25 (66). Two other inhibitory sequences are located further downstream (66). It is unknown whether the inhibitory elements affect initiation or elongation or both in the absence of the positive element.

Two mutations within the first 10 *COX2* codons that reduce translation are suppressed by the dominant *RSM28-1* mutation. The same mutations (*cox2-22* and *cox2-27*) are suppressed by mRNA sequence changes that reduce the stability of a stem structure that begins with the bases of the 10th *COX2* codon (3). It is unclear whether the stronger stems reduce initiation, elongation, or both. The same *cox2* mutations are also suppressed by overproduction of Pet111p and by overproduction of a large-subunit mitochondrial ribosomal protein, MrpL36p (3). MrpL36p contains an essential domain homologous to the bacterial ribosomal protein L31. It also contains an additional partially dispensable domain that, when overexpressed as a protein lacking the L31 region, suppresses the *cox2* mutations but does not detectably associate with ribosomes (67). Interestingly, MrpL36p overexpression also partially suppresses a *cox2* initiation codon mutation but not the corresponding *cox3* mutation (67).

While the mechanism of yeast mitochondrial initiation codon recognition is not understood, it clearly involves both

the initiation codon itself and other features of the mRNA (4, 15, 43). Pet111p and protein components of the ribosome apparently recognize the *COX2* initiation site via interactions with the mRNA 5′-UTL, the initiation codon, and possibly sequences within the coding sequence. Rsm28p could clearly play a positive but nonessential role at this step. We propose that, in the absence of the wild-type sequence of the first 10 *COX2* codons, this process could be antagonized by elements further downstream within the *COX2* coding sequence. Alteration of Rsm28p, increased levels of Pet111p, or increased levels of the non-L31 domain of MrpL36p could at least partially restore translation initiation. (Overexpression of wild-type *RSM28* does not cause suppression.) Such a model would not exclude subsequent effects of the downstream negative elements on translation elongation.

Apparent orthologues of Rsm28p are encoded in the sequenced genomes of other budding yeasts (5, 35) and the filamentous fungus *Ashbya gossypii* (*Eremothecium gossypii*) (6) but are not clearly detectable in more divergent species. Thus, Rsm28p is not highly conserved in sequence and may play a specialized role in fungal mitochondria. While sequence comparisons do not shed light on its function, it is noteworthy that the homologous proteins of *Saccharomyces castellii*, *Saccharomyces kluyveri*, *Kluyveromyces lactis*, and *A. gossypii* lack various extents of the 67-amino-acid nonessential sequence that is deleted by the suppressor mutation *RSM28-1* of *S. cerevisiae*.

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