

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

Elizabeth H. Williams,<sup>†</sup> Nada Bsat, Nathalie Bonnefoy,<sup>‡</sup> Christine A. Butler,  
and Thomas D. Fox\*

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York

Received 17 November 2004/Accepted 6 December 2004

**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<sub>4</sub>Cl. 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<sup>m</sup>* 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).

\* Corresponding author. Mailing address: Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853-2703. Phone: (607) 254-4835. Fax: (607) 255-6249. E-mail: tdf1@cornell.edu.

<sup>†</sup> Present address: Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Md.

<sup>‡</sup> Present address: Centre de Génétique Moléculaire, Laboratoire propre du CNRS associé à l'Université Pierre et Marie Curie, Gif-sur-Yvette, France.

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

Strain	Genotype <sup>a</sup>	Source or reference
DL2	<i>MATa lys2 [rho<sup>+</sup>]</i>	15
DUL1	<i>MATα lys2 ura3Δ [rho<sup>+</sup>]</i>	15
EHW292	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 rsm28Δ::URA3 [rho<sup>+</sup>, cox2(1-91)::ARG8<sup>m</sup>]</i>	This study
EHW385	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 rsm28Δ::URA3 [rho<sup>+</sup>, cox2Δ::ARG8<sup>m</sup>]</i>	This study
EHW424	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 rsm28Δ::URA3 [rho<sup>+</sup>, cox2-22]</i>	This study
EHW463	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox1Δ::ARG8<sup>m</sup>]</i>	This study
EHW464	<i>MATa arg8Δ::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 rsm28Δ::URA3 [rho<sup>+</sup>, cox1Δ::ARG8<sup>m</sup>]</i>	This study
EHW465	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox3Δ::ARG8<sup>m</sup>]</i>	This study
EHW466	<i>MATa arg8Δ::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 rsm28Δ::URA3 [rho<sup>+</sup>, cox3Δ::ARG8<sup>m</sup>]</i>	This study
HMD22	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2Δ::ARG8<sup>m</sup>]</i>	4
LSF74	<i>MATa lys2 ura3Δ [rho<sup>+</sup>, cox3-1]</i>	14
NAB86	<i>MATα ade2-101 ade3-24 rsm28Δ::URA3 leu2-3,112 ura3-52 [rho<sup>+</sup>]</i>	This study
NAB93	<i>MATa ade2-101 ade3-24 arg8::hisG rsm28Δ::URA3 leu2-3 leu2-112 ura3-52 [rho<sup>+</sup>, cox2-22]</i>	This study
NAB97	<i>MATa arg8::hisG RSM28-HA his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-22]</i>	This study
NAB106	<i>MATa arg8::hisG RSM28-1-HA his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-22]</i>	This study
NAB108	<i>MATα ade2-101 ade3-24 rsm28Δ::URA3 leu2-3,112 ura3-52 [rho<sup>+</sup>, cox2-27]</i>	This study
NAB109	<i>MATa arg8Δ::hisG rsm28Δ::URA3 his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-22::ARG8<sup>m</sup>]</i>	This study
NAB111	<i>MATa arg8::hisG rsm28Δ::URA3 his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2::ARG8<sup>m</sup>]</i>	This study
NAB112	<i>MATa arg8::hisG rsm28Δ::URA3 his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-27::ARG8<sup>m</sup>]</i>	This study
NAB119	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2::ARG8<sup>m</sup>]</i>	This study
NAB120	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-22]</i>	This study
NAB121	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-22]</i>	This study
NAB122	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-27]</i>	This study
NAB123	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-22::ARG8<sup>m</sup>]</i>	This study
NAB124	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-27::ARG8<sup>m</sup>]</i>	This study
NAB125	<i>MATα ade2-101 ade3-24 leu2-3,112 ura3-52 pDB20 [rho<sup>+</sup>]</i>	This study
NAB126	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>]</i>	This study
NAB127	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2::ARG8<sup>m</sup>]</i>	This study
NAB128	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-22::ARG8<sup>m</sup>]</i>	This study
NAB129	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-27::ARG8<sup>m</sup>]</i>	This study
NAB130	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 pDB20 [rho<sup>+</sup>, cox2-27]</i>	This study
NB40-3C	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-62]</i>	4
NB43	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2(1-91)::ARG8<sup>m</sup>]</i>	4
NB60	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-10]</i>	4
NB64	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-22]</i>	3
NB64-S7c	<i>MATa arg8::hisG RSM28-1 his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-22]</i>	This study
NB80	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>]</i>	4
NB110	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-10(1-91)::ARG8<sup>m</sup>]</i>	4
NB134	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-34(1-91)::ARG8<sup>m</sup>]</i>	4
NB164	<i>MATa arg8::hisG his3ΔHindIII leu2-3,112 lys2 ura3-52 [rho<sup>+</sup>, cox2-34]</i>	4

<sup>a</sup> 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 congeneric 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  $\lambda$  *cl* 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 ( $\approx$ 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 ( $\approx$ 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 Tn7 mutagenesis was used. The Tn7-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 Tn7, revealing insertion of Tn7 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 (TGATCCTCCCTTGATT) 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 *Dra*I, and subcloned into the *Sma*I site of the multicopy 2 $\mu$ m 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 *Sac*I-*Xba*I 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 *Pac*I fragment from plasmid pJM2 (42) and, for 15S rRNA, *Xho*I-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  $\mu$ g 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*<sub>2</sub>, 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  $\times$  g) and layered onto a 39-ml continuous 15 to 30% sucrose gradient containing 500 mM NH<sub>4</sub>Cl, 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 minitab with 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,

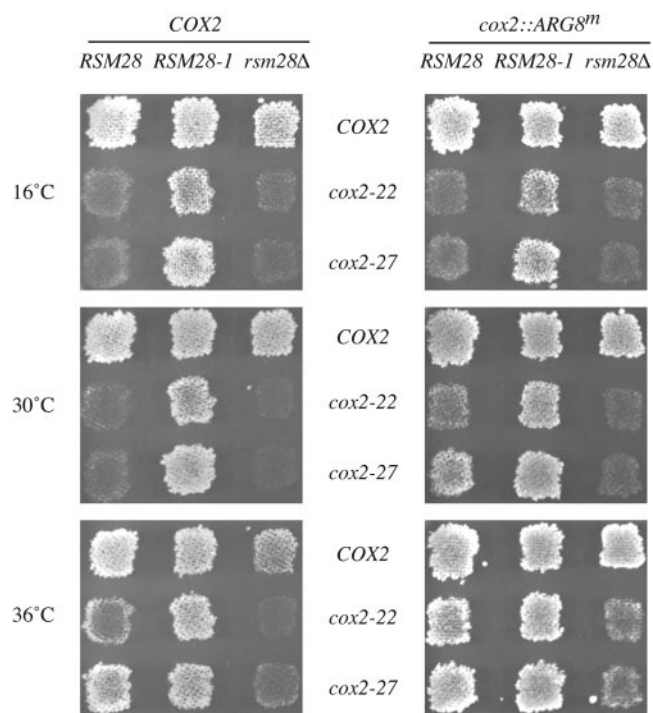


FIG. 1. *RSM28* alleles affect respiratory growth and mitochondrial reporter activity in *cox2-22* and *cox2-27* strains. Strains with the indicated nuclear wild-type *RSM28*, suppressor *RSM28-1*, or deletion *rsm28Δ::URA3* alleles and the indicated mitochondrial alleles in an intact *COX2* locus or the *cox2::ARG8<sup>m</sup>* reporter fusion were grown as patches on minimal glucose medium and then replica plated to non-fermentable minimal medium for the *COX2* alleles (left panels) or minimal glucose medium lacking arginine for the *cox2::ARG8<sup>m</sup>* 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<sup>m</sup>*; NAB119, NAB127, and NAB111; *cox2-22::ARG8<sup>m</sup>*; NAB123, NAB128, and NAB109; and *cox2-27::ARG8<sup>m</sup>*; NAB124, NAB129, and NAB112. As indicated in Table 1, strains lacking a chromosomal *URA3* gene were transformed with the *URA3* plasmid pDB20 (12).

*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).

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

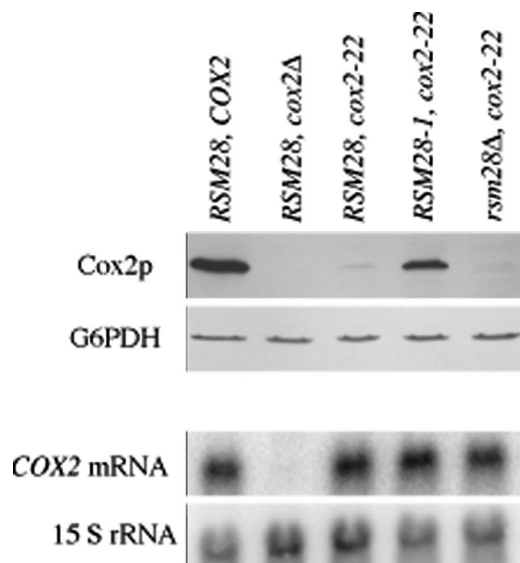


FIG. 2. *RSM28-1* increases Cox2p accumulation in a *cox2-22* mutant without affecting *COX2* mRNA levels. Strains containing wild-type *RSM28*, suppressor *RSM28-1*, or deletion *rsm28Δ::URA3* genomic alleles and wild-type *COX2*, a *cox2* deletion, or *cox2-22* mitochondrial alleles were grown to mid-log 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  $\mu$ g 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  $\mu$ g 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 cox2Δ* (NB40-3C); *RSM28 cox2-22* (NB64); *RSM28-1 cox2-22* (NB64-S7c); and *rsm28Δ cox2-22* (EHW424).

*cox2::ARG8<sup>m</sup>* 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<sup>m</sup>* and *cox2-27::ARG8<sup>m</sup>* 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<sup>-</sup> 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 Tn7 (Materials and Methods). Five mutagenized plasmids that had

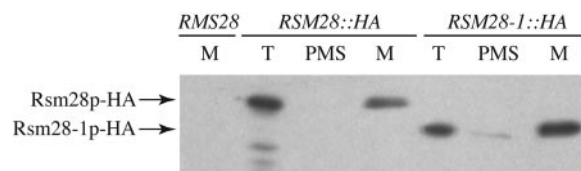


FIG. 3. Rsm28p cofractionates with mitochondria. Cells were grown in YPAGal at 30°C to late log phase, disrupted to yield total cell extracts (T), and fractionated into crude mitochondrial pellets and postmitochondrial supernatants (PMS), and crude mitochondria were then purified on Nycodenz gradients (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.

lost the ability to suppress *cox2-22* were isolated and sequenced, revealing distinct Tn7 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.)

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-

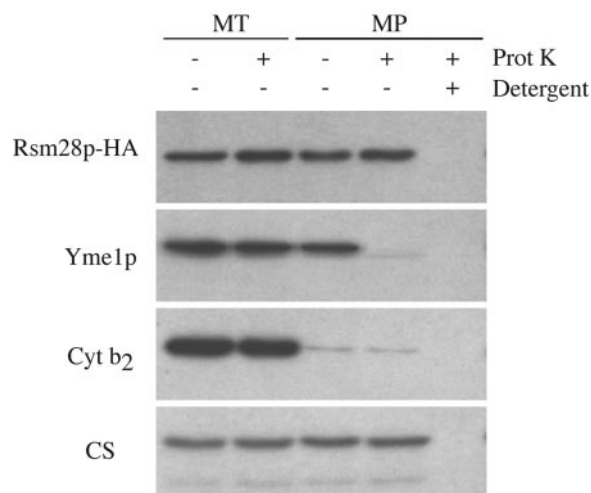


FIG. 4. Rsm28p is within the inner membrane of mitoplasts. Mitochondria (MT) were purified from strain NAB97 (*RSM28::HA*) and converted to mitoplasts (MP) by osmotic shock in the absence or presence of 20  $\mu$ g of proteinase K (Prot K) per ml, as indicated. Mitoplasts also were treated with proteinase 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*<sub>2</sub> as an intermembrane space marker (Cyt *b*<sub>2</sub>), and anti-citrate synthase as a matrix marker (CS).

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  $\text{NH}_4\text{Cl}$  (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

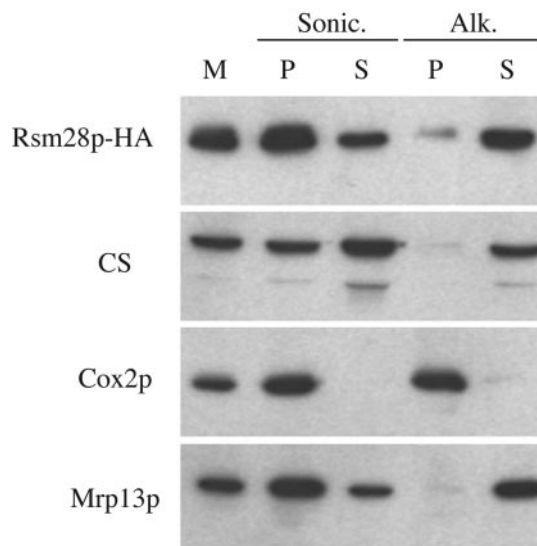


FIG. 5. Rsm28p is peripherally associated with mitochondrial membranes. Mitochondria (M) purified from a strain expressing Rsm28p-HA (NAB97) were sonicated (Sonic.) and separated into a membrane pellet (P) and soluble supernatant (S) by centrifugation. An aliquot of this membrane fraction was extracted 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.

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

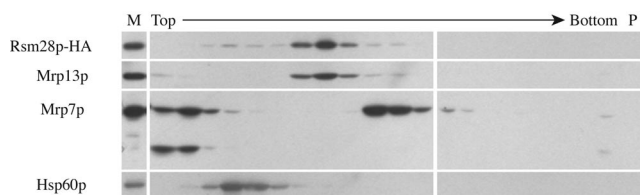


FIG. 6. Rsm28p cosediments with the mitochondrial ribosomal small subunit. Mitochondria were isolated from a strain expressing Rsm28p-HA (NAB97). Mitochondrial ribosomes were extracted by treatment with Triton X-100 and sedimented through continuous 15 to 30% sucrose gradients containing 0.5 M  $\text{NH}_4\text{Cl}$ . 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  $\rho^0$  cells, which cannot assemble ribosomal subunits, was dramatically lower than in  $\rho^+$  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 $\Delta$ ::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<sub>2</sub>O<sub>2</sub> 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<sup>m</sup>* and *cox2-27::ARG8<sup>m</sup>* reporters, *rsm28 $\Delta$ ::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 $\Delta$ ::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 $\Delta$ ::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 $\Delta$ ::URA3* on the expression of two different *cox2::ARG8<sup>m</sup>* reporters: one has *ARG8<sup>m</sup>* 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<sup>+</sup> growth [Fig. 1].) Furthermore, expression of the *ARG8<sup>m</sup>* 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 2 $\mu$ m 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 2 $\mu$ m plasmid suppressed more strongly (Fig. 8A). Wild-type *RSM28* failed to suppress *cox2-22* when carried on either the

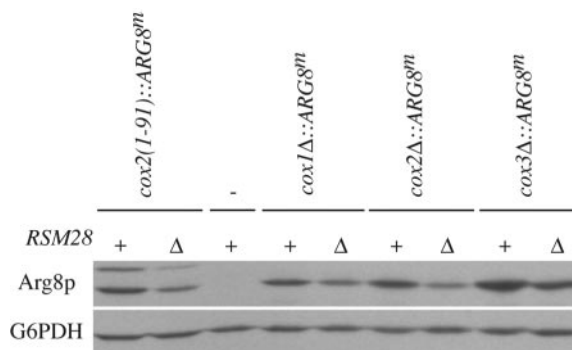


FIG. 7. Rsm28p is required for full expression of the *ARG8<sup>m</sup>* reporter inserted at the *COX1*, *COX2*, and *COX3* loci. Western blots of 50  $\mu$ g of total cellular protein extracted from cells grown to mid- to late log phase in complete medium containing galactose (YPAGal) were probed with anti-Arg8p. The relevant mitochondrial genotypes of each strain are shown above the horizontal lines: - indicates wild-type mitochondrial DNA lacking *ARG8<sup>m</sup>*. The relevant nuclear genotypes are wild-type *RSM28* (+) and the null allele *rsm28 $\Delta$ ::URA3* ( $\Delta$ ). 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 2 $\mu$ m plasmid (Fig. 8A), although this gene did complement the *rsm28 $\Delta$ ::URA3* null allele (unpublished data).

To investigate the allele specificity of suppression by *RSM28-1*, we transformed the high-copy-number 2 $\mu$ m 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 of 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 2 $\mu$ m 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<sup>m</sup>* 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<sup>m</sup>* 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-

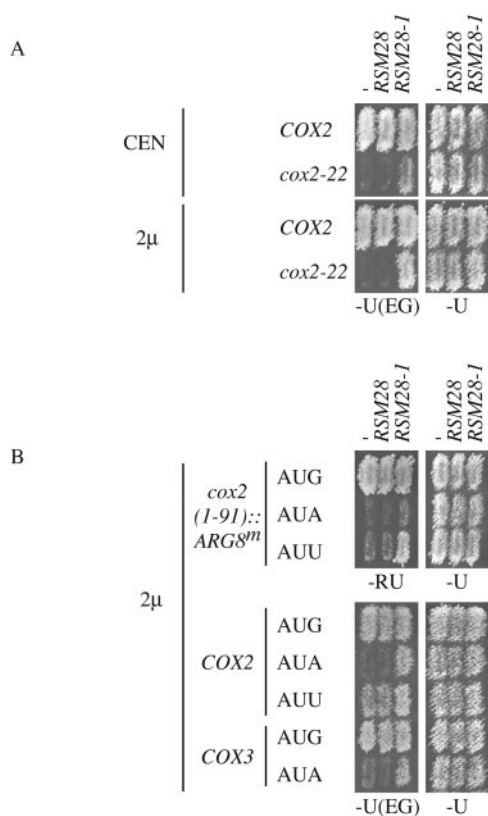


FIG. 8. Plasmid-borne *RSM28-1* can suppresses *cox2-22*, as well as *cox2* and *cox3* initiation codon mutations. (A) *COX2* (NB80) and *cox2-22* (NB64) strains were transformed with centromeric (*CEN*) or  $2\mu$ m multicopy plasmids carrying *RSM28* or *RSM28-1*, as indicated. – indicates empty vector. 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  $2\mu$ m plasmids indicated as –, *RSM28*, and *RSM28-1* were YE352, pEHW217, and pEHW218, respectively (Materials and Methods). (B) Wild-type (AUG) and initiation codon mutant (AUA and AUU) strains were transformed with  $2\mu$ m plasmids expressing *RSM28* or *RSM28-1*. *cox2(1-91)::ARG8<sup>m</sup>* strains (top panels) were replica plated to medium selecting for Arg<sup>+</sup> 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  $2\mu$ m 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 se-

lected 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 *cox1Δ::ARG8<sup>m</sup>* and *cox3Δ::ARG8<sup>m</sup>* 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*.

#### ACKNOWLEDGMENTS

We thank R. L. Hallberg, G. Schatz, B. Glick, T. L. Mason, and C. Koehler for gifts of antibodies.

E. H. Williams was a Howard Hughes Medical Institute Predoctoral Fellow. N. Bonnefoy was a Human Frontier Science Program Organization long-term fellow (LT22/96) during the early phases of this investigation. This study was supported by NIH research grant GM29362 to T.D.F.

#### REFERENCES

- Attardi, G., and G. Schatz. 1988. Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* **4**:289–333.
- Baronas-Lowell, D. M., and J. R. Warner. 1990. Ribosomal protein L30 is dispensable in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:5235–5243.
- Bonnefoy, N., N. Bsat, and T. D. Fox. 2001. Mitochondrial translation of *Saccharomyces cerevisiae COX2* mRNA is controlled by the nucleotide sequence specifying the pre-Cox2p leader peptide. *Mol. Cell. Biol.* **21**:2359–2372.
- Bonnefoy, N., and T. D. Fox. 2000. *In vivo* analysis of mutated initiation codons in the mitochondrial *COX2* gene of *Saccharomyces cerevisiae* fused to the reporter gene *ARG8<sup>m</sup>* reveals lack of downstream reinitiation. *Mol. Gen. Genet.* **262**:1036–1046.
- Cliften, P., P. Sudarsanam, A. Desikan, L. Fulton, B. Fulton, J. Majors, R. Waterston, B. A. Cohen, and M. Johnston. 2003. Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. *Science* **301**:71–76.
- Dietrich, F. S., S. Voegeli, S. Brachat, A. Lerch, K. Gates, S. Steiner, C. Mohr, R. Pohlmann, P. Luedi, S. Choi, R. A. Wing, A. Flavier, T. D. Gaffney, and P. Philippsen. 2004. The *Ashbya gossypii* genome as a tool for mapping the ancient *Saccharomyces cerevisiae* genome. *Science* **304**:304–307.
- Dresios, J., P. Panopoulos, K. Suzuki, and D. Synetos. 2003. A dispensable yeast ribosomal protein optimizes peptidyltransferase activity and affects translocation. *J. Biol. Chem.* **278**:3314–3322.
- Dunstan, H. M., N. S. Green-Willms, and T. D. Fox. 1997. *In vivo* analysis of *Saccharomyces cerevisiae COX2* mRNA 5'-untranslated leader functions in mitochondrial translation initiation and translational activation. *Genetics* **147**:87–100.
- Elble, R. 1992. A simple and efficient procedure for transformation of yeast. *BioTechniques* **13**:18–20.
- Fearon, K., and T. L. Mason. 1992. Structure and function of MRP20 and MRP49, the nuclear genes for two proteins of the 54 S subunit of the yeast mitochondrial ribosome. *J. Biol. Chem.* **267**:5162–5170.
- Fearon, K., and T. L. Mason. 1988. Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies MRP7, a protein of the large subunit of the mitochondrial ribosome. *Mol. Cell. Biol.* **8**:3636–3646.
- Fikes, J. D., D. M. Becker, F. Winston, and L. Guarente. 1990. Striking conservation of TFIIID in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Nature* **346**:291–294.
- Fiori, A., T. L. Mason, and T. D. Fox. 2003. Evidence that synthesis of the *Saccharomyces cerevisiae* mitochondrially-encoded ribosomal protein Var1p may be membrane localized. *Eukaryot. Cell.* **2**:651–653.
- Folley, L. S., and T. D. Fox. 1994. Reduced dosage of genes encoding ribosomal protein S18 suppresses a mitochondrial initiation codon mutation in *Saccharomyces cerevisiae*. *Genetics* **137**:369–379.
- Folley, L. S., and T. D. Fox. 1991. Site-directed mutagenesis of a *Saccharomyces cerevisiae* mitochondrial translation initiation codon. *Genetics* **129**:659–668.
- Fox, T. D. 1996. Genetics of mitochondrial translation, p. 733–758. *In* J. W. B. Hershey, M. B. Matthews, and N. Sonenberg (ed.), *Translational control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Fox, T. D., L. S. Folley, J. J. Mulero, T. W. McMullin, P. E. Thorsness, L. O. Hedin, and M. C. Costanzo. 1991. Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.* **194**:149–165.
- Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93**:97–102.
- Gan, X., M. Kitakawa, K. Yoshino, N. Oshiro, K. Yonezawa, and K. Isono. 2002. Tag-mediated isolation of yeast mitochondrial ribosome and mass spectrometric identification of its new components. *Eur. J. Biochem.* **269**:5203–5214.
- Glick, B. S. 1995. Pathways and energetics of mitochondrial protein import in *Saccharomyces cerevisiae*. *Methods Enzymol.* **260**:224–231.
- Glick, B. S., and L. A. Pon. 1995. Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol.* **260**:213–223.
- Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver. 1996. Life with 6000 genes. *Science* **274**:546, 563–567.
- Graack, H. R., and B. Wittmann-Liebold. 1998. Mitochondrial ribosomal proteins (MRPs) of yeast. *Biochem. J.* **329**:433–448.
- Green-Willms, N. S., C. A. Butler, H. M. Dunstan, and T. D. Fox. 2001. Pet111p, an inner membrane-bound translational activator that limits expression of the *Saccharomyces cerevisiae* mitochondrial gene *COX2*. *J. Biol. Chem.* **276**:6392–6397.
- Green-Willms, N. S., T. D. Fox, and M. C. Costanzo. 1998. Functional interactions between yeast mitochondrial ribosomes and mRNA 5'-untranslated leaders. *Mol. Cell. Biol.* **18**:1826–1834.
- Guthrie, C., and G. R. Fink (ed.). 1991. Guide to yeast genetics and molecular biology. Academic Press, San Diego, Calif.
- Haffter, P., and T. D. Fox. 1992. Suppression of carboxy-terminal truncations of the yeast mitochondrial mRNA-specific translational activator PET122 by mutations in two new genes, *MRP17* and *PET127*. *Mol. Gen. Genet.* **235**:64–73.
- Haffter, P., T. W. McMullin, and T. D. Fox. 1991. Functional interactions among two yeast mitochondrial ribosomal proteins and an mRNA-specific translational activator. *Genetics* **127**:319–326.
- Hallberg, E. M., Y. Shu, and R. L. Hallberg. 1993. Loss of mitochondrial hsp60 function: nonequivalent effects on matrix-targeted and intermembrane-targeted proteins. *Mol. Biol. Cell* **13**:3050–3057.
- Hamel, P., C. Lemaire, N. Bonnefoy, P. Brivet-Chevillotte, and G. Dujardin. 1998. Mutations in the membrane anchor of yeast cytochrome *c*<sub>1</sub> compensate for the absence of Oxa1p and generate carbonate-extractable forms of cytochrome *c*<sub>1</sub>. *Genetics* **150**:601–611.
- He, S., and T. D. Fox. 1997. Membrane translocation of mitochondrially coded Cox2p: distinct requirements for export of amino- and carboxy-terminal, and dependence on the conserved protein Oxa1p. *Mol. Biol. Cell* **8**:1449–1460.
- Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**:163–167.
- Islas-Osuna, M. A., T. P. Ellis, L. L. Marnell, T. M. Mittelmeier, and C. L. Dieckmann. 2002. Cbp1 Is Required for Translation of the Mitochondrial Cytochrome *b* mRNA of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**:37987–37990.
- Jia, L., M. Dienhart, M. Schramp, M. McCauley, K. Hell, and R. A. Stuart. 2003. Yeast Oxa1 interacts with mitochondrial ribosomes: the importance of the C-terminal region of Oxa1. *EMBO J.* **22**:6438–6447.
- Kellis, M., N. Patterson, M. Endrizzi, B. Birren, and E. S. Lander. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**:241–254.
- Koc, E. C., W. Burkhardt, K. Blackburn, A. Moseley, and L. L. Spremulli. 2001. The small subunit of the mammalian mitochondrial ribosome. Identification of the full complement of ribosomal proteins present. *J. Biol. Chem.* **276**:19363–19374.
- Koc, E. C., W. Burkhardt, K. Blackburn, M. B. Moyer, D. M. Schlatter, A. Moseley, and L. L. Spremulli. 2001. The large subunit of the mammalian



- mitochondrial ribosome: Analysis of the complement of ribosomal proteins present. *J. Biol. Chem.* **276**:43958–43969.
38. Krause, K., R. Lopes de Souza, D. G. Roberts, and C. L. Dieckmann. 2004. The mitochondrial message-specific mRNA protectors Cbp1 and Pet309 are associated in a high-molecular weight complex. *Mol. Biol. Cell* **15**:2674–2683.
  39. Li, Y., W. B. Holmes, D. R. Appling, and U. L. RajBhandary. 2000. Initiation of protein synthesis in *Saccharomyces cerevisiae* mitochondria without formylation of the initiator tRNA. *J. Bacteriol.* **182**:2886–2892.
  40. McMullin, T. W., P. Haffter, and T. D. Fox. 1990. A novel small subunit ribosomal protein of yeast mitochondria that interacts functionally with an mRNA-specific translational activator. *Mol. Cell. Biol.* **10**:4590–4595.
  41. Mulero, J. J., and T. D. Fox. 1993. Alteration of the *Saccharomyces cerevisiae* COX2 5'-untranslated leader by mitochondrial gene replacement and functional interaction with the translational activator protein PET111. *Mol. Biol. Cell* **4**:1327–1335.
  42. Mulero, J. J., and T. D. Fox. 1993. PET111 acts in the 5'-leader of the *Saccharomyces cerevisiae* mitochondrial COX2 mRNA to promote its translation. *Genetics* **133**:509–516.
  43. Mulero, J. J., and T. D. Fox. 1994. Reduced but accurate translation from a mutant AUA initiation codon in the mitochondrial COX2 mRNA of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **242**:383–390.
  44. Naithani, S., S. A. Saracco, C. A. Butler, and T. D. Fox. 2003. Interactions among COX1, COX2 and COX3 mRNA-specific translational activator proteins on the inner surface of the mitochondrial inner membrane of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14**:324–333.
  45. O'Brien, T. W., J. Liu, J. E. Sylvester, E. B. Mougey, N. Fischel-Ghodsian, B. Thiede, B. Wittmann-Liebold, and H. R. Graack. 2000. Mammalian mitochondrial ribosomal proteins (4). Amino acid sequencing, characterization, and identification of corresponding gene sequences. *J. Biol. Chem.* **275**:18153–18159.
  46. Partaledis, J. A., and T. L. Mason. 1988. Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies MRP13, a protein of the small subunit of the mitochondrial ribosome. *Mol. Cell. Biol.* **8**:3647–3660.
  47. Pinkham, J. L., A. M. Dudley, and T. L. Mason. 1994. T7 RNA polymerase-dependent expression of COXII in yeast mitochondria. *Mol. Cell. Biol.* **14**:4643–4652.
  48. Reading, D. S., R. L. Hallberg, and A. M. Myers. 1989. Characterization of the yeast HSP60 gene coding for a mitochondrial assembly factor. *Nature* **337**:655–659.
  49. Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**:1030–1032.
  50. Rödel, G. 1997. Translational activator proteins required for cytochrome *b* synthesis in *Saccharomyces cerevisiae*. *Curr. Genet.* **31**:375–379.
  51. Rose, M. D., and J. R. Broach. 1991. Cloning genes by complementation in yeast. *Methods Enzymol.* **194**:195–230.
  52. Sanchirico, M. E., T. D. Fox, and T. L. Mason. 1998. Accumulation of mitochondrially synthesized *Saccharomyces cerevisiae* Cox2p and Cox3p depends on targeting information in untranslated portions of their mRNAs. *EMBO J.* **17**:5796–5804.
  53. Saveanu, C., M. Fromont-Racine, A. Harington, F. Ricard, A. Namane, and A. Jacquier. 2001. Identification of 12 new yeast mitochondrial ribosomal proteins including 6 that have no prokaryotic homologues. *J. Biol. Chem.* **276**:15861–15867.
  54. Schneider, B. L., W. Seufert, B. Steiner, Q. H. Yang, and A. B. Futcher. 1995. Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae*. *Yeast* **11**:1265–1274.
  55. Shen, Z., and T. D. Fox. 1989. Substitution of an invariant nucleotide at the base of the highly conserved "530-loop" of 15S rRNA causes suppression of mitochondrial ochre mutations. *Nucleic Acids Res.* **17**:4535–4539.
  56. Sherman, F., G. R. Fink, and C. W. Lawrence. 1974. *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  57. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
  58. Steele, D. F., C. A. Butler, and T. D. Fox. 1996. Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. *Proc. Natl. Acad. Sci. USA* **93**:5253–5257.
  59. Suzuki, T., M. Terasaki, C. Takemoto-Hori, T. Hanada, T. Ueda, A. Wada, and K. Watanabe. 2001. Proteomic analysis of the mammalian mitochondrial ribosome. Identification of protein components in the 28 S small subunit. *J. Biol. Chem.* **276**:33181–33195.
  60. Suzuki, T., M. Terasaki, C. Takemoto-Hori, T. Hanada, T. Ueda, A. Wada, and K. Watanabe. 2001. Structural compensation for the deficit of rRNA with proteins in the mammalian mitochondrial ribosome. Systematic analysis of protein components of the large ribosomal subunit from mammalian mitochondria. *J. Biol. Chem.* **276**:21724–21736.
  61. Szyrach, G., M. Ott, N. Bonnefoy, W. Neupert, and J. M. Herrmann. 2003. Ribosome binding to the Oxal complex facilitates co-translational protein insertion in mitochondria. *EMBO J.* **22**:6448–6457.
  62. Thorsness, P. E., K. H. White, and T. D. Fox. 1993. Inactivation of YME1, a member of the *ftsH-SEC18-PASI-CDC48* family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:5418–5426.
  63. Tibbetts, A. S., L. Oesterlin, S. Y. Chan, G. Kramer, B. Hardesty, and D. R. Appling. 2003. Mammalian mitochondrial initiation factor 2 supports yeast mitochondrial translation without formylated initiator tRNA. *J. Biol. Chem.*
  64. Vambutas, A., S. J. Ackerman, and A. Tzagoloff. 1991. Mitochondrial translational-initiation and elongation factors in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **201**:643–652.
  65. Wiesenberger, G., M. C. Costanzo, and T. D. Fox. 1995. Analysis of the *Saccharomyces cerevisiae* mitochondrial COX3 mRNA 5'-untranslated leader: translational activation and mRNA processing. *Mol. Cell. Biol.* **15**:3291–3300.
  66. Williams, E. H., and T. D. Fox. 2003. Antagonistic signals within the COX2 mRNA coding sequence control its translation in *Saccharomyces cerevisiae* mitochondria. *RNA* **9**:419–431.
  67. Williams, E. H., X. Perez-Martinez, and T. D. Fox. 2004. MrpL36p, a highly diverged L31 ribosomal protein homolog with additional functional domains in *Saccharomyces cerevisiae* mitochondria. *Genetics* **167**:65–75.
  68. Yaffe, M. P. 1991. Analysis of mitochondrial function and assembly. *Methods Enzymol.* **194**:627–643.