Reduced Dosage of Genes Encoding Ribosomal Protein S18 Suppresses a Mitochondrial Initiation Codon Mutation in *Saccharomyces cerevisiae*

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Manuscript received August 31, 1993
Accepted for publication February 18, 1994

ABSTRACT

A yeast mitochondrial translation initiation codon mutation affecting the gene for cytochrome oxidase subunit III (*COX3*) was partially suppressed by a spontaneous nuclear mutation. The suppressor mutation also caused cold-sensitive fermentative growth on glucose medium. Suppression and cold sensitivity resulted from inactivation of the gene product of *RPS18A*, one of two unlinked genes that code the essential cytoplasmic small subunit ribosomal protein termed S18 in yeast. The two S18 genes differ only by 21 silent substitutions in their exons; both are interrupted by a single intron after the 15th codon. Yeast S18 is homologous to the human S11 (70% identical) and the *Escherichia coli* S17 (35% identical) ribosomal proteins. This highly conserved family of ribosomal proteins has been implicated in maintenance of translational accuracy and is essential for assembly of the small ribosomal subunit. Characterization of the original *rps18a-1* missense mutant and *rps18a*Δ and *rps18b*Δ null mutants revealed that levels of suppression, cold sensitivity and paromomycin sensitivity all varied directly with a limitation of small ribosomal subunits. The *rps18a-1* mutant was most affected, followed by *rps18a*Δ then *rps18b*Δ. Mitochondrial mutations that decreased *COX3* expression without altering the initiation codon were not suppressed. This allele specificity implicates mitochondrial translation in the mechanism of suppression. We could not detect an epitope-tagged variant of S18 in mitochondria. Thus, it appears that suppression of the mitochondrial translation initiation defect is caused indirectly by reduced levels of cytoplasmic small ribosomal subunits, leading to changes in either cytoplasmic translational accuracy or the relative levels of cytoplasmic translation products.

THE accuracy of cytoplasmic translation in the yeast *Saccharomyces cerevisiae* is sensitive to the dosage of some components of the translational machinery. For example, elevated misreading due to increased dosage of translational factors (CHERNOFF *et al.* 1992; SONG *et al.* 1989) or to a shortage of 60S subunits (MORITZ *et al.* 1991) has been reported. In turn, mitochondrial gene expression is dependent on cytoplasmic translational accuracy, as evidenced by omnipotent suppressors in yeast that secondarily cause respiratory deficiency (*e.g.*, KUSHNIROV *et al.* 1988; TER-AVANESYAN *et al.* 1982). In addition, overexpression of two genes, *TIF1* and *TIF2*, which code for yeast cytoplasmic translation initiation factor 4A (eIF-4A), suppresses a missense mutation in the mitochondrial *COX3* gene (LINDER and SLONIMSKI 1989).

While the RNA components (tRNAs, rRNAs) of the mitochondrial translation system and a single ribosomal protein (r-protein) are coded by the mitochondrial genome, all other protein components of the mitochondrial gene expression system are nuclear-coded, cytoplasmically translated, and imported (ATTARDI and SCHATZ 1988; COSTANZO and FOX 1990; DUJON 1981; GRIVELL 1989; TZAGOLOFF and DIECKMANN 1990). Furthermore, some components of the mitochondrial translation system involved in tRNA modification and charging are coded by the same nuclear genes that encode the corresponding cytoplasmic/nuclear proteins (CHATTON *et al.* 1988; CHEN *et al.* 1992; ELLIS *et al.* 1989; GILLMAN *et al.* 1991; NAJARIAN *et al.* 1987; NATSOULIS *et al.* 1986).

In an attempt to identify nuclear-coded components involved in translational start-site recognition in yeast mitochondria (*cf.* DONAHUE *et al.* 1988), we have selected mutations that suppress a mitochondrial initiation codon mutation. The mutation, *cox3-1*, has an AUG-to-AUA change in the mitochondrial gene for cytochrome *c* oxidase subunit III (coxIII) and causes a partial respiratory-deficient phenotype due to decreased translation of coxIII protein (FOLLEY and FOX 1991). In this report, we describe the analysis of one of the suppressors that was of particular interest because it caused a secondary phenotype of cold-sensitive growth on glucose medium. The suppressor mutation was identified as an inactivating mutation in one of two genes that code the essential r-protein S18 in yeast.

MATERIALS AND METHODS

Yeast strains, media and genetic methods: Yeast strains used in this study are listed in Table 1. All strains used were isogenic or congenic to the D273-10B genetic background of *Saccharomyces cerevisiae*, except CSH86L and JRY2659.

Complete media (1% yeast extract, 2% peptone) containing 2% glucose (YPD), or 3% ethanol and 3% glycerol (YPEG), minimal medium (0.67% yeast nitrogen base) with 2% glucose

TABLE 1

Yeast strains used in this study

Strain	Nuclear genotype	Mitochondrial genotype
DUL2	*MATa ura3 lys2*	[ρ^+]
LSF74	*MATa ura3 lys2*	[ρ^+ *cox3-1*]
LSF158	*MATa ura3 lys2 rps18a-1*	[ρ^+ *cox3-1*]
LSF158(pLFY153i)[a]	*MATa ura3 lys2 rps18a-1::pURA3*	[ρ^+ *cox3-1*]
LSF239(pLFY172i)[b]	*MATα ade2 ura3 leu2-3,112 rps18a-1::URA3*	[ρ^+ *cox3-1*]
LSF172	*MATa ade2 ura3 rps18a-1*	[ρ^+ *cox3-1*]
LSF175	*MATα ura3 lys2 rps18a-1*	[ρ^+ *cox3-1*]
LSF325	*MATα ade2 ura3 trp1-1 leu2-3,112 rps18aΔ::URA3*	[ρ^+ *cox3-1*]
LSF326	*MATa ade2 ura3 trp1-1 leu2-3,112 rps18bΔ::LEU2*	[ρ^+ *cox3-1*]
LSF327	*MATα ade2 ura3 trp1-1 leu2-3,112*	[ρ^+ *cox3-1*]
LSF306	*MATα ura3 trp1-1 leu2-3,112 rps18aΔ::URA3 rps18bΔ::LEU2* [pLFY204][c]	[ρ^+ *cox3-1*]
LSF320	*MATα ura3 trp1-1 leu2-3,112 rps18aΔ::URA3 rps18bΔ::LEU2* [pLFY218][d]	[ρ^+ *cox3-1*]
LSF353	*MATa ura3 lys2*	[ρ^+ *cox3-516*][e]
LSF356	*MATa ura3 lys2*	[ρ^+ *cox3-16*][e]
LSF357	*MATa ura3 lys2*	[ρ^+ *cox3-17*][e]
LSF363	*MATa ura3 lys2 rps18a-1*	[ρ^+ *cox3-516*][e]
LSF366	*MATa ura3 lys2 rps18a-1*	[ρ^+ *cox3-16*][e]
LSF367	*MATa ura3 lys2 rps18a-1*	[ρ^+ *cox3-17*][e]
CSH86L[f]	*MATα spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1*	[ρ^+]
PTY21rho^0[g]	*MATa ade2 ura3-52 trp1-1 leu2-3,112*	[ρ^0]
JRY2659[h]	*MATα his3* and/or *his6 leu2-3,112 trp1-289 ura3 cdc7-1*	[ρ^+]

[a] The entire pLFY153i plasmid (895-bp insert in pRS306) is integrated at the *RPS18A* locus.
[b] The *URA3* gene alone is integrated immediately upstream of the *rps18a-1* locus.
[c] pLFY204 is [CEN-*TRP1-RPS18A*].
[d] pLFY218 is [CEN-*TRP1-RPS18A::HA*].
[e] mtDNA donor strains were generously provided by GERLINDE WIESENBERGER (*cox3-516*; unpublished) and MARIA COSTANZO (*cox3-16* and *cox3-17*, unpublished).
[f] CSH86L is from the Cold Spring Harbor collection.
[g] PTY21rho^0 was generously provided by PETER THORSNESS (THORSNESS and FOX 1993).
[h] JRY2659 was generously provided by AMY AXELROD of the JASPER RINE laboratory.

(SD), and sporulation medium (SPO) were as described by SHERMAN *et al.* (1986). Complete media were supplemented with tryptophan for growth of *trp1*$^-$ strains.

Standard genetic procedures were followed (SHERMAN *et al.* 1986). Yeast cells were transformed with a lithium acetate procedure (ITO *et al.* 1983) modified as follows. Cells in exponential growth were harvested, washed in water and resuspended in 0.02 volume of 0.1 M Li-acetate/40% polyethylene glycol (PEG) in 10 mM Tris, 1 mM EDTA (TE), pH 8.0 (Li-acetate/PEG/TE), then incubated at 30° for 2 hr. From 0.1 to 2 μg DNA and 40 μg carrier DNA (herring sperm DNA) were added to 100 μl of concentrated cells. After further incubation for 30 min, 400 μl of the Li-acetate/PEG/TE solution at 30° were added, and the tube rolled or inverted at 30° for 1 hr. Cells were collected by centrifugation (2–5 sec in a microcentrifuge), washed in SD medium containing 1 M sorbitol and resuspended in the same medium to a volume appropriate for plating on one to three plates.

Nucleic acid manipulation and analysis: Standard techniques were employed for restriction digests, cloning, gel electrophoresis and gel blot analyses (SAMBROOK *et al.* 1989). The *RPS18A* and *RPS18B* sequences have been entered in GenBank as accession nos. L15408 and L17004, respectively. The *RPS18A* locus was marked to facilitate mapping by integrating a cut plasmid (pLFY153i) composed of an 895-base pair *Dra*I-*Dra*I fragment of the 3′ portion of *RPS18A* inserted into the vector pRS306 (*URA3*; SIKORSKI and HIETER 1989). In addition, in both a wild-type and an *rps18a-1* suppressor strain, the *URA3* gene was inserted into flanking sequences upstream of the *RPS18A* locus (*Cla*I site at nucleotide (nt) 175; by integration of a pLFY172i fragment). The phenotypes of the wild-type and suppressor strains were unaffected by this integration.

Marked null mutations were created in *RPS18A* and *RPS18B* by single-step gene disruption of the chromosomal copies (ROTHSTEIN 1983). A total deletion/disruption of *RPS18A* replaced sequence from upstream of the promoter to downstream of the stop codon with *URA3* (by integration of a pLFY176i fragment; nt 175 *Cla*I to nt 1494 *Sna*BI was deleted). A partial deletion/disruption of *RPS18A* replaced the TATA box, first exon and part of the intron with *URA3* (with a pLFY177i fragment; nt 515 *Sty*I to nt 824 *Eco*RI deleted). The phenotypes of these two *RPS18A* deletions were indistinguishable. The partial deletion/disruption of *RPS18B* replaced the tripartite promoter homologies, first exon, intron, and part of the second exon with *LEU2* (by integration of a pLFY188i fragment; nt 300 *Cla*I to nt 1366 *Bgl*II deleted). Gel blot analyses (SOUTHERN 1975) were used to confirm the presence of the marker genes at the proper loci (data not shown).

Sequencing on double-stranded DNA templates employed the chain termination method of SANGER *et al.* (1977) and the reagents of the Sequenase v. 2.0 kit (U.S. Biochemical Corp.). Sequence reconstructions and analyses, including nucleotide and protein homology searches, were done using the programs of the Genetics Computer Group (1991). The GenBank, EMBL, and Swiss-Prot databases were searched.

Construction of epitope-tagged S18: An oligonucleotide was synthesized (5′-TCTTGGAAGTCTTAACAGCGTAGTCTGGGACGTCGTATGGGTACTTTGGGTTGTTGAAGAT) to insert the sequence of the 12CA5 epitope from influenza hemagglutinin (KOLODZIEJ and YOUNG 1991) into the *RPS18A* sequence after codon 24 by site-directed mutagenesis (Muta-

gene, Bio-Rad). Putative mutants were screened by restriction digest, using the diagnostic *Aat*II site of the epitope. The tagged gene *RPS18A::HA*, confirmed by sequence analysis, was recloned as a 1.7-kb fragment into the *Bam*HI-*Sal*I sites of a CEN vector with a *TRP1* selectable marker (pRS314; SIKORSKI and HIETER 1989) to create pLFY218. (pLFY204 carries the same fragment of the wild-type, untagged, *RPS18A* gene.)

Isolation of crude cytoplasmic and mitochondrial ribosomal fractions for blotting: Cytoplasmic and mitochondrial ribosomal fractions were isolated from 6–7.5 liters of cells grown to late exponential phase. Spheroplasts were prepared, resuspended in 0.6 M mannitol, 1 mM EDTA (pH 6.8), and disrupted (MCMULLIN *et al.* 1990). The cell homogenate was clarified twice by centrifugation (3,000 rpm, 5 min in SS-34 rotor), then mitochondria were pelleted at 12,000 rpm for 15 min. Cytoplasmic ribosomes were pelleted from the postmitochondrial supernatant by spinning at 27,000 rpm in a Beckman SW41 rotor (~90,000 × *g*) for 1 hr, resuspended in mannitol/EDTA and then clarified by centrifugation at 10,000 rpm for 10 min. Mitochondria were resuspended in mannitol/EDTA, clarified by centrifugation (3,500 rpm, 5 min), washed 5–6 times in mannitol/EDTA, clarified again and pelleted. The pellets were resuspended in 50 mM KCl, 10 mM Mg-acetate, 10 mM Tris (pH 7.5), 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (resuspension buffer, modified from previous methods (GRIVELL *et al.* 1971; SINGH *et al.* 1978), and mitochondria were lysed after 10 min on ice by the addition of 0.1 volume of 10% sodium deoxycholate. Lysates were clarified at 15,000 rpm, 20 min, and ribosomes were pelleted from the supernatant at 34,000 rpm in an SW41 rotor (~143,000 × *g*) for 90 min. The pellet was gently rinsed three times with resuspension buffer, resuspended in the same solution and clarified at 10,000 rpm for 10 min.

Immunoblots of ribosomal fractions: Samples were run on 15% polyacrylamide-0.1% SDS, Tris-glycine gels, blotted to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore), and probed with primary antisera as follows: rabbit anti-PET123 (MCMULLIN *et al.* 1990) at 1:5000 dilution (treated with acetone powder as in HARLOW and LANE, 1988); rabbit anti-L16 (a generous gift from J. L. WOOLFORD) at 1:2500 dilution; and protein A-purified monoclonal anti-HA from 12CA5 cells (KOLODZIEJ and YOUNG 1991; BAbCO, Berkeley California) at a 1:7200 dilution of the 2.5 mg/ml stock. Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Bethesda Research Laboratories), or HRP-conjugated goat anti-mouse (Sigma) at 1:30000 dilution. ECL chemiluminescence detection reagents (Amersham Corp.) and Kodak XAR-5 film were used to visualize antibody binding.

Cytoplasmic polyribosome preparation and analysis: Cells were grown at 30°, or were shifted to 18° for 3 hr, prior to harvest at an OD_{600} ~0.8. Polyribosomes captured by cycloheximide treatment were extracted from cells lysed with glass beads and separated on linear 7 to 47% sucrose gradients as described by BAIM *et al.* (1985), except that lysing by 15-sec vortex: 45 sec on ice was continued for 30 cycles to increase the fraction of cells broken. Forty A_{254} units were layered onto 36-ml gradients and centrifuged in an SW28 rotor at 22,000 rpm for 4 hr, 26 min at 4°. Absorbances at 254 nm were read and recorded while gradients were pumped through a Beckman DU-65 spectrophotometer.

Paromomycin sensitivity: Cells were suspended in water in a microtiter plate and replica-spotted (with a multi-prong device) onto YPD agar plates that contained tryptophan and either 100, 400 or 800 μg/ml paromomycin sulfate. Plates were incubated for 2 days at 30°.

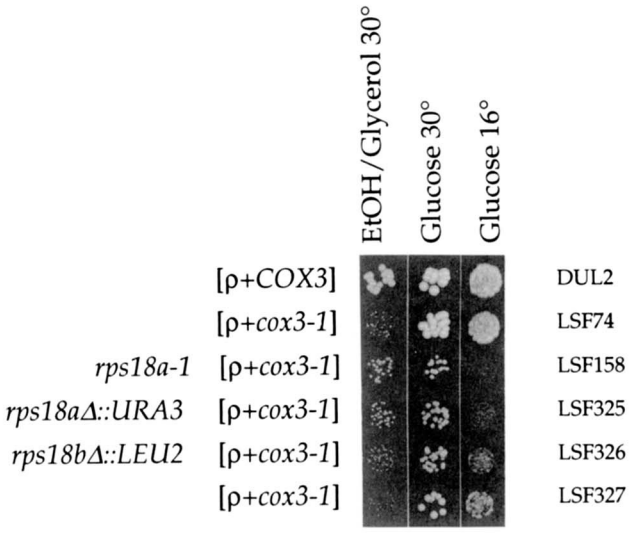

FIGURE 1.—Mutations affecting *RPS18* genes cause suppression of the mitochondrial mutation *cox3-1* and cold-sensitive growth on medium containing glucose. Cells, serially diluted in water, were spotted onto plates containing the nonfermentable carbon sources ethanol and glycerol (YPEG) or glucose (YPD; both media were supplemented with tryptophan), and incubated at either 16° or 30° as shown. Incubation was 2 days for YPD at 30°, 5 days for YPEG at 30°, 6 days for YPD at 16°. Strains and relevant genotypes are given.

RESULTS

A suppressor of a mitochondrial initiation codon mutation that caused cold-sensitive growth on glucose: The AUG-to-AUA initiation codon mutation, *cox3-1*, produces a partial respiratory-deficient phenotype (FOLLEY and FOX 1991). Spontaneous revertants were selected on nonfermentable YPEG medium at 30°. Several revertants carried nuclear suppressor mutations that caused temperature-sensitive growth defects on glucose (suppression and temperature sensitivity cosegregated in meiotic crosses), suggesting that the wild-type alleles of the suppressor genes were required for functions other than mitochondrial gene expression (L. S. FOLLEY and T. D. FOX, manuscript in preparation).

One such suppressor mutation, termed *rps18a-1* for reasons described below, improved respiratory growth at 30°, but virtually blocked growth on rich glucose medium (YPD) at 16° (Figure 1). Expression of coxIII protein was not detectably affected by this suppressor (as judged by *in vivo* labelings of mitochondrial translation products in the presence of cycloheximide; data not shown). This result suggests that this weak suppression may require only a small change in coxIII expression, and/or that suppression works through an indirect mechanism. The cold-sensitive growth phenotype on YPD was expressed both in *rps18a-1* mutant strains containing wild-type mtDNA and those lacking mtDNA entirely (*rho*^{0}; not shown), and is therefore independent of mitochondrial genotype.

Isolation, sequencing, identification and mapping of the *RPS18A* and *RPS18B* genes: Plasmids capable of

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Human      1 MADIQTERAYQKQPTIFQNKKRVLLGETGKEKLPRYYKNIGLGFKTPKEAIE 52
             :|:|||:|||| || | |       |:| :  |:||| |||||||| |||
Yeast      1 .STELTVQSERAFQKQPHIFNNPK....VKTSK.RTKRWYKNAGLGFKTPKTAIE 50

Human     53 GTYIDKKCPFTGNVSIRGRILSGVVTKMKMQRTIVIRRDYLHYIRKYNRF 102
             |:|||||||||| |||||:||:| |   ||:|||||| ||||| ||||:
Yeast     51 GSYIDKKCPFTGLVSIRGKILTGTVVSTKMHRTIVIRRAYLHYIPKYNRY 100
                       : : : | | ||| || ::||:    :   | | ::
E. coli                1 TDKIRTLQGRVVSDKMEKSIVVAIERFVKHPIYGKF  36

Human     103 EKRHKNMSVHLSPCFRDVQIGDIVTVGECRPLSKTVRFNVLKVTKAAG.T 151
              ||||||: ||:|| || ||:|||||||:|||:|||||||||:||: |||
Yeast     101 EKRHKNVPVHVSPAFR.VQVGDIVTVGQCRPISKTVRFNVVKVSAAAGKA 149
              ||   : ||          :||:| : :|||:|||  : :|:|   |
E. coli    37 IKRTTKLHVHDENN..ECGIGDVVEIRECRPLSKTKSWTLVRVVEKAVL   83

Human     152 KKQFQKF 158
              ||| ||
Yeast     150 NKQFAKF 156
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FIGURE 2.—Amino acid sequence homologies of ribosomal protein S18 of yeast with human S11 and *E. coli* S17. | = amino acid identity. : = amino acid similarity. The *rps18a-1* mutation is N to K at position 138 (shown in boldface). Yeast S18 is 70% identical to human S11, and 35% identical to *E. coli* S17. Human sequence from LOTT and MACKIE (1988) and *E. coli* sequence from YAGUCHI and WITTMANN (1978) and ZURAWSKI and ZURAWSKI (1985) *via* the Swiss-Prot database, which was accessed through the GCG system (Genetics Computer Group 1991). Homologies were detected by the FASTA program (PEARSON and LIPMAN 1988).

complementing the *rps18a-1* cold-sensitive growth phenotype were selected by transforming the suppressor strain LSF158 with a YCp50-based yeast genomic bank (ROSE *et al.* 1987). The cold-resistant transformants each contained one of two non-overlapping chromosomal regions on their plasmids. Sequence analysis of the complementing DNA in both classes of plasmids revealed two virtually identical genes coding for the yeast homolog of the human (LOTT and MACKIE 1988) and rat (TANAKA *et al.* 1985) cytoplasmic r-protein termed S11, and *Escherichia coli* r-protein S17 (YAGUCHI and WITTMANN 1978; ZURAWSKI and ZURAWSKI 1985). The yeast protein is 70% identical to human S11 and 35% identical to *E. coli* S17 (Figure 2).

A comparison of the predicted amino-terminal peptide sequence to peptide sequences determined for a number of yeast r-proteins (TAKAKURA *et al.* 1992) identified the product of these genes as the yeast cytoplasmic r-protein called variously rp41 (TAKAKURA *et al.* 1992; WARNER and GORENSTEIN 1978), YS12 (OTAKA and OSAWA 1981) and S18 (KRUISWIJK and PLANTA 1974; reviewed by RAUE *et al.* 1991). S18 is acetylated on the N-terminal serine (TAKAKURA *et al.* 1992). It is a basic protein (calculated pI = 11.55) with a calculated molecular mass of 20.5 kDa. Following the convention established for other yeast r-protein gene pairs, we have named these genes *RPS18A* and *RPS18B* (the DNA sequences are not presented here; GenBank accession nos. are L15408 and L17004, respectively).

To confirm that the cloned *RPS18A* DNA corresponded to the original suppressor mutation, the marker *URA3* was inserted into cloned sequences upstream of *RPS18A* (pLFY172i; see MATERIALS AND METHODS). A linear fragment containing *URA3* flanked by cloned DNA was used to transform a cold-sensitive, Sup$^+$ *rps18a-1* strain by homologous double recombination. The resulting transformant had *URA3* tightly linked to the cold-sensitive *rps18a-1* mutation; the diploid strain formed by mating to wild-type PTY21rho^0 produced parental ditypes in all 10 tetrads scored. *In vivo* repair of a gapped CEN plasmid (ORR-WEAVER *et al.* 1983) bearing *RPS18A*-flanking DNA permitted isolation of the suppressor mutation *rps18a-1*: a C to A change at codon 138. This mutation changes an asparagine to lysine, and apparently inactivates the protein (see below).

RPS18A was mapped by tetrad analysis to chromosome *IV* (R), 4.5 centimorgans from *TRP1* (Table 2). The gene for seryl-tRNA synthetase, *SES1* (formerly *SERS*, EMBL accession no. X0884; KOLMAN *et al.* 1988; WEYGAND-DURASEVIC *et al.* 1987), was found in the DNA sequence immediately upstream of the *RPS18A* gene (Figure 3). The *RPS18B* gene had been previously identified in a partial sequence (J. WARNER, personal communication) of a DNA fragment carrying *REB1*, a gene located on chromosome *II* (R) (JU *et al.* 1990; Figure 3).

RPS18A and _RPS18B_ are a conventional r-protein gene pair: The genes *RPS18A* and *RPS18B* are similar in structure to many r-protein gene pairs that have been characterized in yeast (reviewed in WOOLFORD and WARNER 1991; WOOLFORD 1991). Both genes have a single intron that splits a small (15-codon) first exon from a larger (141-codon) second exon (Figure 3). Characteristically, the coding sequences are highly homologous; they differ at 21 positions, but code identical amino acid sequences. The introns (339 base pairs in *RPS18A*; 512 base pairs in *RPS18B*) and flanking regions of the two genes share no obvious homology. Both *RPS18A* and *RPS18B* have the tripartite promoter structure–the RPG, HOMOL1 and T-rich sequences–characteristic of r-protein genes [Figure 3; LEER *et al.* (1985) and ROTENBERG and WOOLFORD (1986); reviewed in WOOLFORD (1991)]. A DNA gel-blot hybridization analysis that

TABLE 2

Tetrad analysis of genetic linkage between markers in the *RPS18A* region on chromosome *IV*

Interval	PD	NPD	TT	Distance (cM)
cdc7-trp1[a]	59	1	11	12
trp1-rps18a[a,b,c]	146	0	13	4.5
cdc7-rps18a[a]	55	2	14	18

[a] Cross of LSF158(pLFY153i) × JRY2659.
[b] Cross of LSF172 × CSH86L.
[c] Cross of LSF175 × PTY21rho^0.

used *RPS18A* second exon sequences as a probe under stringent conditions indicated that there are only two genes for S18 (data not shown).

A possible mitochondrial-specific first exon sequence beginning upstream of *RPS18A* appeared to be consistent with the possibility that S18 was a shared protein. This sequence starts 120 base pairs downstream of the *SES1* stop codon (about 14 nt upstream of the *Cla*I site, Figure 3; position 161 of the *RPS18A* sequence; GenBank no. L15408) and overlaps the *RPS18A* promoter. It could code a sequence of 81 amino acids that has properties similar to mitochondrial targeting signals (VON HEIJNE 1986). The presence of a near-consensus 5′ splice site sequence suggested that this exon potentially could be spliced to the second exon of *RPS18A* upon removal of a 638-base intron. However, we could not detect transcripts copied from this putative exon, and its disruption (at the *Cla*I site with a fragment of pLFY172i) had no detectable phenotypic consequences (data not shown).

Inactivation of *RPS18A* causes suppression and cold sensitivity: To determine the null phenotypes of the S18 genes, deletion/disruption alleles were constructed *in vitro*. The entire coding sequence (*Cla*I to *Sna*BI; Figure 3), or the TATA, first exon, and part of the intron sequence (*Sty*I to *Eco*RI; Figure 3) at the *RPS18A* locus were replaced by *URA3* (*rps18aΔ::URA3*); and the promoter, first exon, intron and first half of the second exon of *RPS18B* (*Cla*I to *Bgl*II; Figure 3) were deleted and replaced by *LEU2* (*rps18bΔ::LEU2*). These null alleles were transformed into strains carrying the *rho*$^+$, *cox3-1* mitochondrial genome. The gross phenotypes of the total and partial *rps18aΔ* deletion mutations were indistinguishable (data not shown). Deletion of *RPS18A* caused a cold-sensitive growth phenotype and partial suppression of *cox3-1*–similar to, but slightly weaker than, those phenotypes of the *rps18a-1* spontaneous mutation (Figure 1). Thus, we concluded that these phenotypes are likely due to a reduction of functional S18 in the cells. The *rps18bΔ* mutation caused slight cold-sensitivity for growth on glucose (compared to isogenic wild-type) and only marginally improved the respiratory growth of *cox3–1* mutants (Figure 1), suggesting that more S18 is made by the *RPS18A* gene than by the *RPS18B* gene (and see below).

We confirmed that both *RPS18A* and *RPS18B* are active genes, by demonstrating that spores carrying null mutations in both genes were inviable (data not shown). Strains carrying the single mutations *rps18aΔ::URA3* (partial deletion) and *rps18bΔ::LEU2* were mated and sporulated. Among 100 tetrads analyzed, one-fourth of the spores were inviable, and were predicted by marker segregation to be phenotypically Ura$^+$/Leu$^+$. When the doubly-heterozygous diploid was transformed prior to sporulation with a CEN-*TRP1* plasmid carrying *RPS18A* (pLFY204), Ura$^+$/Leu$^+$/Trp$^+$ spores were recovered (data not shown), demonstrating that inviability had been caused by lack of S18.

An epitope-tagged form of S18 was not detectable in mitochondrial ribosomes or in mitochondria. One possible mechanism of suppression not yet ruled out was the idea that S18 was a shared protein–a component of both mitochondrial and cytoplasmic ribosomes. To attempt to test whether a product of the *RPS18* genes was directed to mitochondria, we created an allele of *RPS18A* that encoded an epitope-tagged form of S18. To minimize possible negative effects of the tag on protein structure and function, the sequence of the 9-amino acid influenza hemagglutinin epitope (HA; KOLODZIEJ and YOUNG 1991) was inserted into the *RPS18A* sequence at site corresponding to a 4-amino acid gap in the homology between the yeast S18 and human S11 (after the 24th codon; Figure 2). To make a strain that was dependent on the epitope-tagged gene, a diploid heterozygous for both *rps18aΔ::URA3* (the partial deletion) and *rps18bΔ::LEU2* was transformed to Trp$^+$ with pLFY218, which carries the tagged *RPS18A* gene. Viable spores that contained both chromosomal disruptions and the plasmid were recovered after sporulation of the transformed diploid. Both fermentative and respiratory growth of doubly disrupted, epitope-tagged plasmid-containing spore clones (*e.g.*, LSF320) were indistinguishable from isogenic strains that contained a plasmid with the wild-type *RPS18A* gene (*e.g.*, LSF306), indicating that the cytoplasmic and mitochondrial ribosomes were functional in the tagged strain.

Immunological examination of r-proteins from strain LSF320 with the 12CA5 monoclonal antibody (KOLODZIEJ and YOUNG 1991) revealed an HA epitope-specific signal corresponding to a protein of approximately 18 kD in cytoplasmic, but not mitochondrial, r-proteins (Figure 4, left). Probing the same blot with antiserum raised against a known mitochondrial r-protein (PET123; HAFFTER *et al.* 1990; MCMULLIN *et al.* 1990) showed that a *bona fide* mitochondrial r-protein was easily detected in this experiment (Figure 4, center).

Approximately equal amounts of pelleted cytoplasmic and mitochondrial ribosomes were loaded in the lanes shown in Figure 4. Only after a long overexposure was a trace of signal observed in the tagged mitochondrial

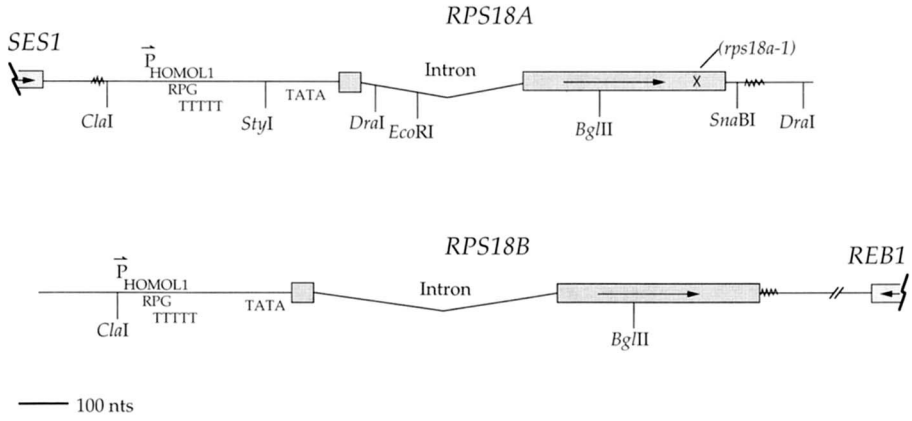

FIGURE 3.—Structures of *RPS18A* and *RPS18B* genes. The drawings are to scale. Protein coding regions are shown as stippled boxes; arrows indicate direction of transcription. Putative poly(A) addition signals are indicated by a zigzag. Only restriction sites mentioned in the text are shown. The first exons of *RPS18A* and *RPS18B* are 15 codons and the second exons are 141 codons long. The position of the *rps18a-1* lesion is indicated. Both genes have the HOMOL1, RPG, and T-rich promoter sequences (LEER *et al.* 1985; ROTENBERG and WOOLFORD 1986). The *REB1* gene (JU *et al.* 1990) is estimated to end within 500 bp from the end of *RPS18B*. The *RPS18A* and *RPS18B* sequences have been entered in GenBank as accession nos. L15408 and L17004, respectively.

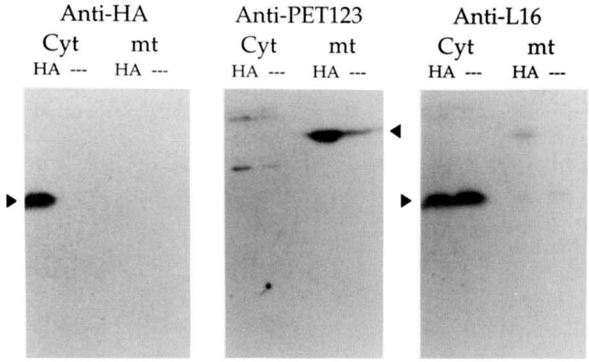

FIGURE 4.—Tagged S18 protein from *RPS18A::HA* is a component of cytoplasmic ribosomes, but is not detectable in mitochondrial ribosomes. Crude intact cytoplasmic ribosomes (Cyt) and mitochondrial ribosomes (mt) from epitope-tagged strain LSF320 (HA) and strain LSF306 (---) were isolated as described in MATERIALS AND METHODS. Approximately 40 μg protein (except as described below) were loaded per lane of the 15% polyacrylamide-0.1% SDS Tris-glycine gel. The blot was probed first with 12CA5 monoclonal antibody (Anti-HA; left), stripped and probed with anti-PET123 polyclonal antiserum (center), then stripped and probed with anti-L16 polyclonal antiserum (right). The bands representing these proteins are indicated by arrowheads. The difference in the PET123 signals between the two strains probably reflects a difference in protein loading, since staining of the gel revealed 2–5-fold less protein in the LSF306 mitochondrial lane than in the LSF320 mitochondrial lane.

lane with the 12CA5 antibody (data not shown). We estimate this signal to be approximately 1/600th as strong as that from the cytoplasmic ribosomes. Assuming the stoichiometry of the putative mitochondrial S18 in ribosomes were similar to that of the cytoplasmic S18, we would have expected their HA-specific signals to be comparable. The trace of signal that we do see is more than accounted for by slight contamination of the mitochondrial ribosome preparation with cytoplasmic r-proteins. This low level of contamination was revealed when the same blot was probed with antiserum raised against the cytoplasmic r-protein L16 (Figure 4, right; MORITZ *et al.* 1990, 1991). One could argue that the epitope-tagged form of S18 might be more loosely associated with mitochondrial ribosomes than the wild-type protein and thus might be lost during ribosome purification. However, we were unable to detect a protease-resistant, tag-specific signal in intact mitochondria (data not shown) suggesting the protein simply is not present in the organelles.

Strains with mutations in S18 genes have reduced ratios of small to large ribosomal subunits: By analogy to bacterial S17, it is likely that S18 is critical for assembly of the small ribosomal subunit (HELD *et al.* 1974; HERZOG *et al.* 1979). To determine the effects of the reduced dosage of the S18 protein on the yeast translational machinery, polyribosome profiles were examined for the mutant and wild-type strains grown at 30° or shifted to 18°. Compared to wild type, all three mutants had substantially reduced levels of 40S subunits, as evidenced by overaccumulation of 60S subunits (Figure 5). [Accumulation of large subunits independently of small subunits has been documented for other small subunit mutants (ABOVICH *et al.* 1985; DONOVAN *et al.* 1990; WOOLFORD 1991).] At the semi-permissive temperature of 18°, the original suppressor mutation, *rps18a-1*, caused the most severe overaccumulation of 60S subunits followed by *rps18aΔ* and *rps18bΔ*; a progression that follows that of both suppression and cold-sensitive growth of these mutants (Figure 1). The results at 30° were similar (Figure 5, lower panels). These data indicate that S18 is essential for the assembly of the small ribosomal subunit, and that more of the S18 protein is made from the *RPS18A* gene than from the *RPS18B* gene. Furthermore, the fact that the *rps18a-1* missense mutation has a more severe phenotype than a deletion of the gene indicates the nonfunctional missense protein may compete with wild-type

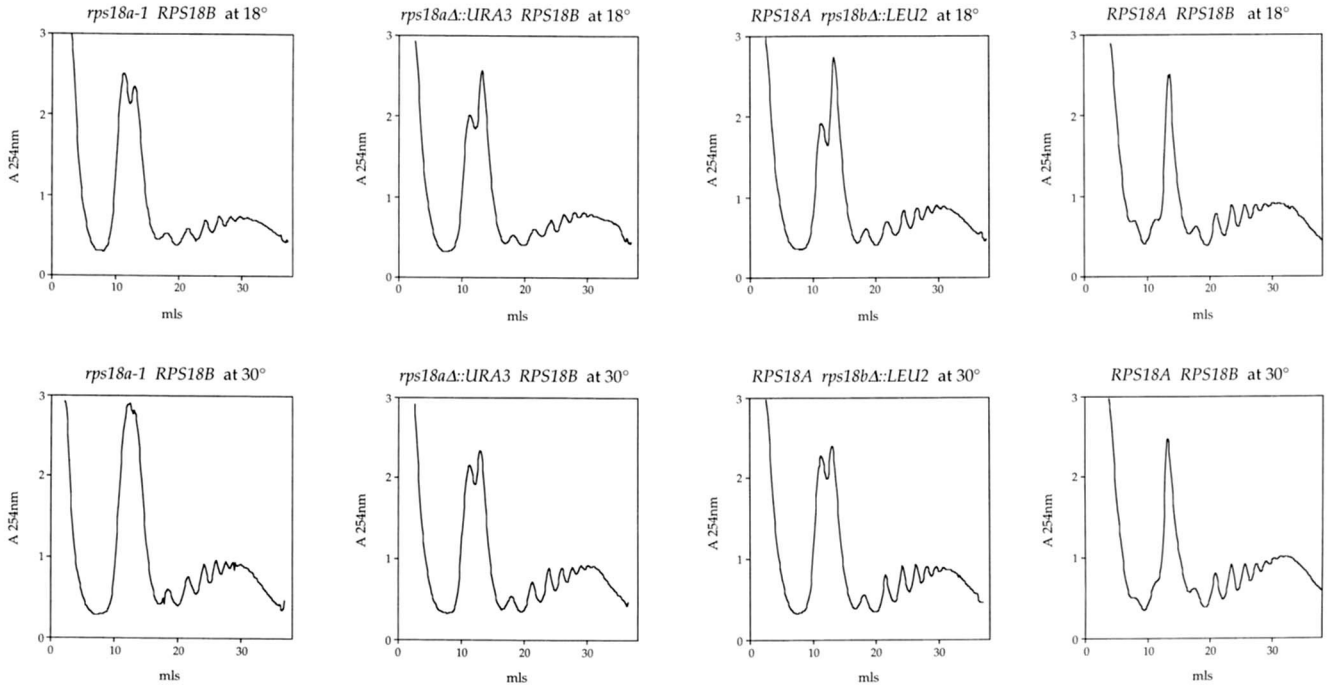

FIGURE 5.—Sucrose gradient profiles of polyribosomes from *rps18* mutant and wild-type strains. Polyribosomes captured by cycloheximide were resolved by sucrose gradient sedimentation as described in MATERIALS AND METHODS. Sedimentation is from left to right. Data shown are absorbances at 254 nm. The four upper traces are data from cells shifted to 18° for 3 hr prior to harvesting polyribosomes. Data from the same strains grown at 30° are shown in the four lower traces. The 40S subunit peak (a shoulder significant only in wild type) is approximately 9 ml from the top of the gradient, the 60S peak is ~11 ml, and the 80S peak (the largest peak in wild type and the right-hand peak of the major doublet in the mutant strains) is approximately 13 ml from the top. Strains shown are: *rps18a-1*, LSF158; *rps18aΔ::URA3*, LSF325; *rps18bΔ::LEU2*, LSF326; wild type, LSF327. (All strains shown are [ρ^+ *cox3-1*].)

S18 for binding to some component necessary for small subunit assembly.

Reduced dosage of S18 increases sensitivity to paromomycin: Wild-type and mutant strains were tested for sensitivity to the aminoglycoside antibiotic paromomycin, a drug that increases the frequency of misreading in yeast (PALMER *et al.* 1979; SINGH *et al.* 1979). Strains with genetic defects lowering fidelity are hypersensitive to the drug (EUSTICE *et al.* 1986; MASUREKAR *et al.* 1981), as are strains with reduced levels of 40S ribosomal subunits (FABIAN and HOPPER 1987; LEE *et al.* 1992; reviewed in WOOLFORD 1991). The paromomycin sensitivities of the mutant strains grown on glucose were found to vary directly with the limitation of 40S subunits, with *rps18a-1* significantly more sensitive than *rps18aΔ*, which in turn was more sensitive than *rps18bΔ* (Figure 6). The sensitivity of an *rps18aΔ*, [ρ^0] strain was similar to that of the *rps18aΔ*, [rho$^+$, *cox3-1*] strain, indicating that mitochondrial gene expression was not required for this phenotype (data not shown).

To assay for an effect on the fidelity of the cytoplasmic ribosomes, we tested whether the *rps18aΔ* mutation could suppress the *lys2-187* allele, a nonsense mutation that is suppressed by many omnipotent suppressors (CHERNOFF *et al.* 1992; EUSTICE *et al.* 1986). We found that it could not (data not shown), which indicated that the effects of 40S subunit limitation on translational fidelity must differ, quantitatively or qualitatively, from that caused by characterized omnipotent suppressors.

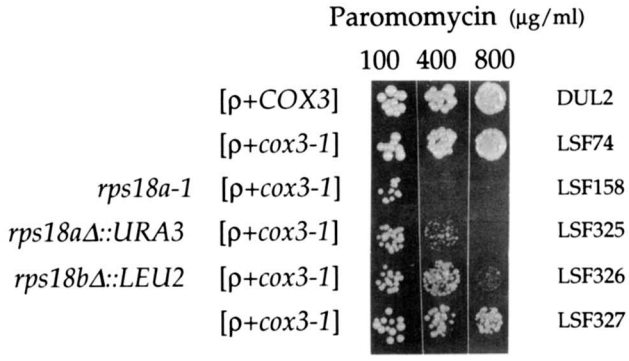

FIGURE 6.—*rps18* mutants are sensitive to paromomycin. Cells suspended in water were spotted on YPD plates (supplemented with tryptophan) containing 100, 400 or 800 μg/ml paromomycin sulfate. The plates were then incubated 2 days at 30°. Strains and relevant genotypes are shown.

Paromomycin was found to phenotypically suppress the *cox3–1* mutation in a wild-type nuclear background (data not shown). However, this result could be due either to cytoplasmic misreading or mitochondrial misreading since paromomycin also acts directly on mitochondrial ribosomes (LI *et al.* 1982; WEISS-BRUMMER and HÜTTENHOFER 1989) and can phenotypically suppress mitochondrial mutations (DUJARDIN *et al.* 1984). In ei-

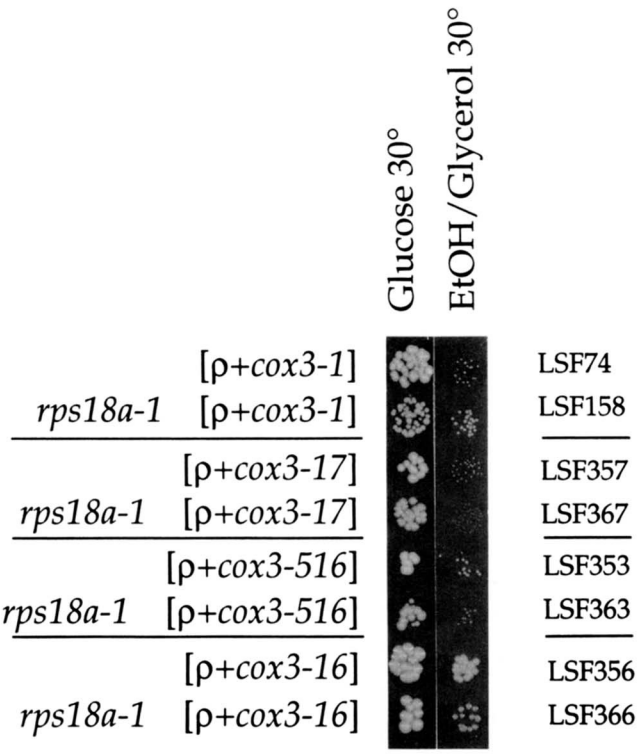

FIGURE 7.—Allele specificity of suppression by *rps18a-1*. Cells suspended in water were spotted onto plates containing ethanol and glycerol (YPEG) or glucose (YPD; both supplemented with tryptophan) and incubated at 30° for 5 days. Relevant genotypes and strain names are indicated.

ther case, this result suggests that a relaxation of translational fidelity is sufficient to suppress the *cox3-1* mutation.

***rps18a-1* suppression is allele-specific, not gene-specific:** To further characterize the suppression mechanism, we tested whether *rps18a-1* could suppress other leaky mitochondrial mutations (Figure 7). An AUA initiation codon mutation in the *COX2* gene (MULERO and FOX 1993) was suppressed by *rps18a-1*, indicating that suppression is not gene-specific (data not shown). However, three deletion mutations affecting the *COX3* mRNA 5′-untranslated leader that cause leaky respiration-deficient growth (comparable to that of *cox3-1*) were not suppressed by *rps18a-1*. Indeed the presence of the *rps18a-1* nuclear mutation had a negative effect on respiratory growth of strains carrying these leaky *cox3* mutations (Figure 7). Thus, suppression of the initiation codon mutations is apparently not due to physiological compensation of low levels of cytochrome oxidase.

DISCUSSION

We have identified and characterized a pair of expressed genes, *RPS18A* and *RPS18B*, that code the essential r-protein S18 in yeast. This gene pair is typical of those previously described (reviewed in WOOLFORD and WARNER 1991; WOOLFORD 1991), with highly homologous coding regions specifying a single protein of 20.5 kD. Under our growth conditions, *RPS18A* appears to be more highly expressed than *RPS18B*.

Yeast S18 is a member of a conserved family of r-proteins with known homologs in mammals (human and rat S11), plants (soybean and Arabidopsis S11), eubacteria (*E. coli* S17), archaebacteria (*Halobacterium marismortui* HmS14) and chloroplasts (Arabidopsis and pea CS17) (Figure 2; ARNDT 1990; GANTT and THOMPSON 1990; KIMURA and KIMURA 1987; LOTT and MACKIE 1988; TANAKA *et al.* 1985; YAGUCHI and WITTMANN 1978; ZURAWSKI and ZURAWSKI 1985). The yeast S18 amino acid sequence is 70% identical to human S11 and 35% identical to *E. coli* S17 (Figure 2).

What is known about the function of the proteins in this family is inferred from studies of S17 in bacteria. S17 is apparently situated near the interface of the large and small subunits (BRIMACOMBE *et al.* 1988; STERN *et al.* 1988; STÖFFLER-MEILICKE *et al.* 1985) and binds discrete 5′, central and 3′ portions of the small rRNA (WIENER *et al.* 1988) with a primary binding site at the 5′ end of 16S (MACKIE and ZIMMERMAN 1978; WEITZMANN *et al.* 1993). Structural analysis of the *Bacillus stearothermophilus* protein indicates the presence of three loops projecting from a twisted five-stranded β-pleated sheet (GOLDEN *et al.* 1993). Loops 1 and 3 probably bind different sites on the 16S rRNA, thereby helping to hold the 16S rRNA in a functional conformation (GOLDEN *et al.* 1993). A neamine-resistance mutation in loop 1 produces ribosomes that are hyperaccurate *in vivo* and *in vitro* (BOLLEN *et al.* 1975; YAGUCHI and WITTMANN 1978) suggesting that the effect of S17 on rRNA conformation is important for the maintenance of translational fidelity.

A bacterial mutation in loop 3 was found to be heat-sensitive for small subunit assembly (HERZOG *et al.* 1979) demonstrating the *in vivo* role of S17 in this process. Consistent with a similar role in eukaryotes, our yeast mutants deficient in the homologous protein S18 contained fewer small subunits than wild type. Interestingly, the missense mutation *rps18a-1* (N to K at position 138) reduces small subunit levels more than the deletion mutation, suggesting that that mutant protein is able to bind nonproductively to 18S rRNA and partially compete with the functional product of the *RPS18B* gene. By analogy to the S17 structure determined by GOLDEN *et al.* (1993), the *rps18a-1* missense mutation appears to change a residue in the fifth β-strand close to loop 3.

The fact that S18 deficiency partially suppressed mitochondrial translation initiation codon mutations suggested the interesting possibility that S18 might be present in both cytoplasmic and mitochondrial ribosomes. However, our inability to detect an epitope-tagged S18 protein in mitochondria or mitochondrial ribosomes argues against a direct role for S18 in mitochondrial protein synthesis.

Our data are most consistent with the hypothesis that partial suppression of mitochondrial initiation codon

mutations is an indirect result of lowered levels of cytoplasmic small ribosomal subunits, although the mechanism is unclear. The fact that lowered S18 levels lead to paromomycin sensitivity suggests the possibility that the accuracy of cytoplasmic translation may be reduced (EUSTICE *et al.* 1986; MASUREKAR *et al.* 1981). This phenotype has previously been associated with reduced levels of mature 40S cytoplasmic ribosomal subunits (FABIAN and HOPPER 1987; LEE *et al.* 1992). Alterations in the levels of other translation system components are known to cause translation elongation errors resulting in informational suppression (CHERNOFF *et al.* 1992; SONG *et al.* 1989) and can affect initiation as well (MORITZ *et al.* 1991). Since the protein components of the mitochondrial translational machinery are, with a single exception, made on cytoplasmic ribosomes, error-prone synthesis of these components could result in relaxed stringency of mitochondrial translation initiation. However, if lowered levels of 40S ribosomal subunits do reduce translational accuracy, the effect is not strong enough to cause suppression of *lys2-187*, an easily suppressed nonsense mutation (CHERNOFF *et al.* 1992; EUSTICE *et al.* 1986).

An alternative indirect mechanism for suppression of mitochondrial mutations could be based on the hypothesis that a deficit of cytoplasmic ribosomal small subunits alters the relative levels of cytoplasmically translated components of the mitochondrial protein synthesis systems (by affecting mRNA competition for cytoplasmic ribosomes). The resulting imbalances could decrease the stringency of mitochondrial translation initiation leading to partial suppression.

Regardless of the mechanism, suppression by *rps18a-1* appears to operate on the mutant initiation codons themselves since it is allele-specific: AUA initiation codon mutations affecting both the *COX3* and *COX2* mRNAs are suppressed, but deletion mutations affecting the *cox3* untranslated leader that confer comparable respiratory growth defects are not suppressed. This result argues strongly against the notion that suppression is caused by physiological changes that partially compensate for the reduced levels of coxIII protein and/or cytochrome oxidase activity caused by the initiation codon mutation(s).

We gratefully acknowledge JON WARNER for helpful discussions and sharing of unpublished data, JOHN WOOLFORD for his gift of antiserum and for stimulating discussions, and BARBARA GOLDEN for sending her preprint prior to publication. We thank TOM MASON for his gift of antiserum, FRED WINSTON for providing a control strain with a tagged gene, and JULIO MULERO, GERLINDE WIESENBERGER and MARIA COSTANZO for providing strains. During a portion of this work, L.S.F. was a Postdoctoral Fellow of the Jane Coffin Childs Memorial Fund for Medical Research and was partially supported by the Fund. This work was supported by a Research Grant from the National Institutes of Health (GM29362) to T.D.F.

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Communicating editor: A. G. HINNEBUSCH