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Functional Interactions between Yeast Mitochondrial Ribosomes and mRNA 5′ Untranslated Leaders

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Translation of mitochondrial mRNAs in *Saccharomyces cerevisiae* depends on mRNA-specific translational activators that recognize the 5′ untranslated leaders (5′-UTLs) of their target mRNAs. We have identified mutations in two new nuclear genes that suppress translation defects due to certain alterations in the 5′-UTLs of both the *COX2* and *COX3* mRNAs, indicating a general function in translational activation. One gene, *MRP21*, encodes a protein with a domain related to the bacterial ribosomal protein S21 and to unidentified proteins of several animals. The other gene, *MRP51*, encodes a novel protein whose only known homolog is encoded by an unidentified gene in *S. kluyveri*. Deletion of either *MRP21* or *MRP51* completely blocked mitochondrial gene expression. Submitochondrial fractionation showed that both Mrp21p and Mrp51p cosediment with the mitochondrial ribosomal small subunit. The suppressor mutations are missense substitutions, and those affecting Mrp21p alter the region homologous to *E. coli* S21, which is known to interact with mRNAs. Interactions of the suppressor mutations with leaky mitochondrial initiation codon mutations strongly suggest that the suppressors do not generally increase translational efficiency, since some alleles that strongly suppress 5′-UTL mutations fail to suppress initiation codon mutations. We propose that mitochondrial ribosomes themselves recognize a common feature of mRNA 5′-UTLs which, in conjunction with mRNA-specific translational activation, is required for organellar translation initiation.

While mitochondrial ribosomes exhibit distinct similarities to bacterial (eubacterial) ribosomes (30, 69), the yeast mitochondrial translation system has many intriguing differences from bacterial systems. Mitochondrial ribosomes have more proteins than do bacterial ribosomes (23, 43). Of the mitochondrial ribosomal proteins whose sequences are known, some are simple homologs of their bacterial counterparts, others have domains homologous to bacterial ribosomal proteins attached to domains with no recognizable homology to any known proteins, and still others are completely unrelated to bacterial ribosomal proteins (reviewed in reference 32). *Saccharomyces cerevisiae* mitochondrial mRNAs generally have long, A+U-rich 5′ untranslated leaders (5′-UTLs) lacking a typical Shine-Dalgarno sequence (11, 27, 31). While the mechanism of start site selection remains obscure in this system, translation initiation on most or all yeast mitochondrial mRNAs requires membrane-bound mRNA-specific activator proteins whose targets lie in the 5′-UTLs (reviewed in reference 27). These mRNA-specific activators appear to play a dual role in mitochondrial gene expression: tethering the synthesis of the very hydrophobic mitochondrial gene products to the inner membrane (27) and modulating the translation levels of individual mRNAs (63).

We have focused on translation of the *COX2* and *COX3* mRNAs, which encode subunits II and III of cytochrome *c* oxidase, respectively. Previous studies have identified their mRNA-specific translational activators and established functional interactions among activator proteins, their mRNA targets, and other components of the mitochondrial translation system. The *COX2*-specific translational activator protein is encoded by the nuclear gene *PET111* (46, 54), while the *COX3*-specific activator is a complex containing three proteins encoded by the nuclear genes *PET54*, *PET122*, and *PET494* (6, 9, 14, 38). Using suppressor analysis, we have shown that one subunit of the *COX3*-specific activator, Pet122p, interacts functionally with the small subunit of mitochondrial ribosomes (33, 35, 42) and that each translational activator interacts functionally with the 5′-UTL of its target mRNA (12, 45, 67). These findings suggested that yeast mitochondrial ribosomes were unable to recognize mRNAs unless the ribosome-mRNA interaction was mediated by translational activators that recognized sites unique to each mRNA.

Here we report that certain mutations in the *COX2* and *COX3* mRNA 5′-UTLs that are suppressible by alterations of mRNA-specific activators can also be suppressed by mutations in nuclear genes encoding two mitochondrial ribosomal small-subunit proteins. The suppression is allele specific, indicating that the ribosomes play an active role in the recognition of translation start signals. Surprisingly, however, suppression is not gene specific, indicating that the ribosomes are recognizing features of the 5′-UTLs that are common to at least several mRNAs. One of these yeast mitochondrial ribosomal proteins is homologous to bacterial S21 and to the products of unidentified genes from several animals.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The *S. cerevisiae* strains used in this study are listed in Table 1. All the strains were isogenic or congenic to the wild-type strain D273-10B (ATCC 25657). The media and genetic methods used were as described previously (60). Respiratory growth was assessed on YPEG medium (3% ethanol, 3% glycerol, 1% yeast extract, 2% Bacto Peptone, 2% agar). Second-site suppressors of 5′-UTL mutations were selected in the strains JJM120, JJM156, MCC199, and MCC200 (Table 1).

Plasmid manipulations, nucleotide sequencing, and computer analysis. Plasmids were constructed and transformed into *Escherichia coli* DH5αF′IQ by standard techniques (58). Nucleotide sequencing was performed either with the Sequenase version 2.0 DNA-sequencing kit (U.S. Biochemicals) or by DNA Services, Cornell University, with an ABI 371 DNA sequencer. Nucleotide sequence data were analyzed with Lasergene biocomputing software (DNAStar, Inc.). The Basic Local Alignment Search Tool (BLAST) (2) program was accessed through the National Center for Biotechnology Information or the *Sac-*

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TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Source or reference
DAU1	*MAT*α *ade2 ura3* [*rho*$^+$]	41
DL1	*MAT*α *lys2* [*rho*$^+$]	45
JJM113	*MAT***a** *lys2 ura3* [*rho*$^+$ *cox2-10*]	47
JJM120	*MAT*α *lys2* [*rho*$^+$ *cox2-11*]	45
JJM156	*MAT*α *lys2 ura3* [*rho*$^+$ *cox2-11*]	45
JJM158	*MAT*α *lys2 ura3* [*rho*$^+$ *cox2-12*]	45
JJM173	*MAT*α *MRP51-5 lys2 ura3* [*rho*$^+$ *cox2-11*]	44
LSF75	*MAT*α *lys2 ura3* [*rho*$^+$ *cox3-1*]	26
MCC199	*MAT*α *lys2* [*rho*$^+$ *cox3-15*]	This study
MCC200	*MAT*α *ade2* [*rho*$^+$ *cox3-15*]	12
MCC211	*MAT*α *MRP21-1 ade2 ura3* [*rho*$^+$ *cox3-15*]	This study
MCC267	*MAT*α *MRP21-1 MRP51-3* [*rho*$^+$ *cox2-11*]	This study
MCC291	*MAT*α *MRP21-HA-TRP1 ura3-52 trp1-*Δ*1* [*rho*$^+$]	This study
NSG50	*MAT*α *MRP51-3 lys2* [*rho*$^+$ *cox2-11*]	This study
NSG59	*MAT***a** *MRP51-3 ade2 ura3* [*rho*$^+$ *cox2-10*]	This study
NSG63	*MAT***a** *mrp51*Δ::*URA3 lys2 ura3*Δ [*rho*^{0}]	This study
NSG78	*MAT*α *MRP51-3 lys2* [*rho*$^+$]	This study
NSG83	*MAT*α *MRP51-3 lys2* [*rho*$^+$ *cox3-1*]	This study
PTY11	*MAT*α *ura3-52 trp1-*Δ*1* [*rho*$^+$]	P. E. Thorsness
PTY22rho0	*MAT***a** *ura3-52 ade2 leu2-3,112* [*rho*^{0}]	P. E. Thorsness
TF210	*MAT***a** *ura3-52 leu2-3,112* [*rho*$^+$ *cox3-15*]	12

[a] Mitochondrial genotypes are in brackets; genes not in brackets are nuclear.

charomyces Genome Database to search for nucleotide and protein sequence similarities.

Cloning of the *MRP21* and *MRP51* genes. Nuclear DNA from a strain carrying both the *MRP21-1* and *MRP51-3* suppressor genes (MCC267 [Table 1]) was prepared as described previously (28) and partially digested with *Sau*3AI; the partial digestion products were separated by size in a 10 to 40% sucrose gradient as described by Rose and Broach (56), and 6- to 10-kb fragments were pooled. The genomic DNA fragments were ligated to *Bam*HI-cleaved YEp24 (5) to create a library of approximately 20,000 independent *E. coli* transformants, which was amplified by standard methods (56).

To clone *MRP21-1*, the *cox3-15* mutant strain TF210 (Table 1) was transformed with the MCC267 library. Transformants were selected on minimal medium and then printed to YPEG medium and incubated at 13.5°C. The transformants that grew on YPEG medium at 13.5°C were analyzed to verify that cold resistance segregated with the plasmid. Six plasmids carrying a region of the genome near the *ROX3* gene and 13 plasmids carrying *PET494* were isolated (see Results). To determine whether *MRP21* was on the plasmids carrying the *ROX3* region, a 4.4-kb *Bam*HI-*Eco*RI fragment from this region was subcloned from plasmid pBSROX3BR (57) into the integrating vector YIp5 (64), which carries the *URA3* gene, creating the plasmid pMC327. pMC327 was cut at a single *Xba*I site in the insert and integrated into the genome of strain TF210 by transformation and homologous recombination; the integrant strain was crossed to the *MRP21-1 cox3-15* strain MCC211, and respiratory growth of the meiotic progeny was analyzed.

To determine the nucleotide sequences of the *MRP21* suppressor alleles, the *MRP21* coding sequence was PCR amplified from genomic DNA of strains carrying each of the suppressor alleles. The nucleotide sequence of the entire PCR product from each strain was determined, and in each case a single nucleotide difference from the wild-type sequence (GenBank accession no. Z35851) was observed. *MRP21-1* and *MRP21-2* alleles had the same mutation, a G-to-A change at nucleotide 343 of the *MRP21* coding region. In the *MRP21-3* allele position 363 of the *MRP21* coding sequence was changed from C to G.

To clone *MRP51-3*, the *cox2-12* mutant strain JJM158 was transformed with the MCC267 library. The transformants were selected on minimal medium and then printed to YPEG medium. Plasmids were isolated from respiratory-competent transformants and transformed back into *cox2-12* and *cox2-11* strains to verify that suppression was plasmid linked. Fourteen overlapping plasmids were isolated. To determine whether *MRP51* was on the suppressing plasmids, a 1,975-bp *Sal*I fragment, including 276 bp of the vector, YEp24, was subcloned from the suppressing library plasmid pB-14 into the *Bam*HI site of the integrating vector pRS306 (62), creating plasmid pNSG17. pNSG17 was cut at either a unique *Afl*II site or a unique *Mun*I-*Mfe*I site in the insert and used for integrative transformation of an *MRP51-5*/*MRP51 cox2-11* diploid strain (JJM173 × PTY22rho0). The integrant strain was sporulated, and the meiotic progeny were analyzed.

The sequence of *MRP51-3* was determined by direct sequencing of the suppressing library plasmid. It corresponded to the wild-type sequence (YPL118W; coordinates 16771 to 17805 of GenBank no. U43503) except for the single nucleotide change from C to G at position 782 of the coding sequence. The other *MRP51* suppressor alleles (except *MRP51-8*) were cloned by gap repair (52), and the ability of gap-repaired plasmids to suppress *cox2-11* was confirmed. For *MRP51-2* and *MRP51-4*, the sequence of the entire open reading frame was determined, partly from the gap-repaired plasmids and partly from PCR products amplified from the genomic DNAs of the suppressor strains; for *MRP51-1* and *MRP51-5*, the sequence of the entire gene except the 3′ 114 bp was determined in the same manner. For *MRP51-8*, the sequence was determined from PCR products amplified from genomic DNA. In *MRP51-1*, position 704 was changed from T to C; in *MRP51-2*, position 721 was changed from A to C; *MRP51-4* had the same change as the independently isolated *MRP51-3* allele (see above); in *MRP51-5*, position 779 was changed from C to T; and in *MRP51-8*, positions 835 and 836 were changed from GA to AG.

In vivo labeling of mitochondrial translation products. In vivo labeling was performed as described previously (28). Cells were grown in galactose-containing minimal medium containing the ^{35}S-labeled *E. coli* hydrolysate labeling reagent Tran ^{35}S-label (ICN Radiochemicals) in the presence of cycloheximide. Crude mitochondria were subjected to electrophoresis on 16% polyacrylamide gels (prepared from a stock solution containing 29.2% acrylamide and 0.8% bisacrylamide) containing 10% glycerol in the presence of 0.1% sodium dodecyl sulfate. The gels were dried and autoradiographed.

Generation of null alleles, and epitope tagging of Mrp21p. An *mrp21* null allele was generated by removing a 459-bp *Cla*I-*Bgl*II fragment internal to the structural gene and inserting a *hisG*::*URA3*::*hisG* cassette (1). An *mrp51* null allele was generated by removing an internal 550-bp *Mun*I-*Bgl*II fragment and inserting the same cassette. To tag the *MRP21* gene with three copies of the sequence encoding the influenza virus hemagglutinin (HA) epitope (25, 65) at its 3′ end, we used the plasmid pCS124 (59), an integrative plasmid carrying three copies of the HA sequence and the *TRP1* gene. The 3′ 294 bp of *MRP21* was amplified by PCR and inserted into pCS124 in frame with the HA sequence. The resulting plasmid, pMC343, was cut at a unique *Eco*RI site within the *MRP21* coding sequence, and the linearized DNA was used to transform strain PTY11 to Trp$^+$. In the resulting integrative transformant, MCC291, the only complete copy of *MRP21* was the tagged allele, *MRP21-HA*.

Mitochondrial isolation and fractionation. Mitochondria were prepared from cells grown to late exponential phase in complete medium (yeast-peptone [YP] medium) containing 2% galactose as described previously (29), except that spheroplasts were disrupted with a Parr-Bomb (Parr Instrument Co., Moline, Ill.) as described previously (17). Mitochondria were purified by equilibrium density gradient centrifugation in Nycodenz [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N*,*N*′-bis(2,3-dihydroxypropyl)-isophthalimide; Sigma, St. Louis, Mo.] step gradients as described previously (29). Mitochondrial ribosomes were analyzed by sucrose gradient centrifugation (15 to 30% sucrose in 0.5 M NH_4Cl, 10 mM Tris [pH 7.4], 10 mM magnesium acetate, 7 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg each of the protease inhibitors antipain, aprotinin, bestatin, chymostatin, E-64, leupeptin, pepstatin A, and phosphorhamidon per ml) directly from disrupted mitochondria as described previously (53). The clarified lysate obtained from 2 mg of whole mitochondria was applied to a 36-ml gradient; 1-ml fractions were collected, and 0.2 ml of each was precipitated with trichloroacetic acid and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels.

Antisera and immunological methods. Mouse monoclonal antibodies against Mrp7p (24) and Mrp13p (53) were obtained from T. L. Mason. Mouse monoclonal antibody 12CA5 against the HA epitope was purchased from BAbCo (Berkeley, Calif.).

Anti-Mrp51p polyclonal rabbit antiserum was prepared as described previously (36) with histidine-tagged Mrp51p as an antigen. An *MRP51* gene with six His codons at the 3′ end of the coding sequence was generated by PCR and inserted into pQE-30 (Qiagen), with an additional six His codons added to the 5′ end of the coding sequence. The resulting plasmid, pNSG29, was transformed into *E. coli* and induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the fusion protein was affinity purified with Ni-nitrilotriacetic acid resin as directed by the manufacturer (Qiagen) (37).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed by standard techniques (36). Antigen-antibody complexes were visualized by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G or goat anti-rabbit immunoglobulin G (Gibco BRL, Bethesda, Md.) secondary antibody and the enhanced chemiluminescence system (Amersham Life Science Inc., Arlington Heights, Ill.).

RESULTS

Selection of second-site suppressors of *cox2* and *cox3* 5′-UTL mutations. The *cox2-11* mutation is a single-base deletion

(deletion of the G residue at −24) in the *COX2* 5′-UTL that does not affect the stability of the *COX2* mRNA but greatly decreases its translation, causing weak respiratory growth (45). In a previous study, six independent dominant nuclear suppressors of the *cox2-11* mutation were isolated (45). One of these affected *PET111*, the known specific translational activator for the *COX2* mRNA, while the other five did not (45). To characterize further the unknown mutations, a strain carrying one of the suppressors was crossed to each of the other four suppressor strains. Analysis of the respiratory growth of the meiotic progeny showed that the first suppressor was tightly linked to each of the other four. Thus, all five *cox2-11* suppressors were in a single gene, now called *MRP51* (*MRP51-1*, *MRP51-2*, *MRP51-3*, *MRP51-4*, and *MRP51-5*). Respiratory growth of the *cox2-11* strains containing these suppressors remained dependent upon the function of the *PET111* gene.

The *cox3-15* mutation, which consists of two deletions in the *COX3* 5′-UTL, causes cold-sensitive respiratory growth (12). The respiratory defect is due to cold-sensitive translation, since the *cox3-15* mRNA is present at wild-type levels in cells grown in the cold but Cox3p is not synthesized (12). Translation of the *cox3-15* mRNA at the permissive temperature is still dependent on the *COX3*-specific translational activator complex (12).

Six spontaneous cold-resistant revertants of a *cox3-15* mutant strain were isolated previously (12), and for this study an additional 20 revertants were isolated. The revertant strains were characterized genetically as previously described (12) to determine whether the dominant suppressor mutations were nuclear or mitochondrial and whether they were linked to any known genes whose products are involved in translational activation. Of the 26 revertants analyzed, 17 had nuclear suppressor mutations. Nine of these suppressors mapped to the *PET122* gene, which encodes a subunit of the *COX3*-specific translational activator (10, 12, 38); three mapped to a new gene we have called *MRP21* (*MRP21-1*, *MRP21-2*, and *MRP21-3*); and one mapped to the *MRP51* gene (*MRP51-8*), also identified above as a suppressor of the *cox2-11* mutation (the four remaining nuclear suppressors were relatively weak and were not studied further). The wild-type function of the *COX3*-specific translational activators *PET54*, *PET122*, and *PET494* was required for respiratory growth of the *cox3-15* strains carrying *MRP21* or *MRP51* suppressor alleles. Thus, the selection of suppressors of 5′-UTL mutations that specifically blocked the translation of particular mitochondrial mRNAs yielded not only mutations in the corresponding specific translational activators (12, 45) but also mutations in two previously unidentified genes whose products interact functionally with the same regions of these 5′-UTLs.

Specificity of the *MRP21* and *MRP51* suppressors. To characterize the specificity of the two novel suppressors, they were combined with a variety of nuclear and mitochondrial mutations affecting translation and the double-mutant phenotypes were analyzed. Respiratory growth, a phenotype we have repeatedly found to be a sensitive indicator of Cox2p and Cox3p synthesis in mutant strains (6, 12, 13, 22, 26, 45), was assessed for all combinations of alleles (Tables 2 and 3; Fig. 1). To confirm that the observed respiratory growth phenotypes reflected the synthesis rates of mitochondrial proteins, in vivo labeling of mitochondrial translation products in the presence of cycloheximide was performed for selected strains (Fig. 2).

None of the suppressor alleles had a detectable phenotype when combined with the wild-type mitochondrial genome. However, some suppressor alleles at both *MRP21* and *MRP51* suppressed some 5′-UTL mutations in both *COX2* and *COX3* (Tables 2 and 3; Fig. 1 and 2), suggesting that Mrp21p and Mrp51p are involved in the translation of at least these two mitochondrial mRNAs.

TABLE 2. Allele specificity of the *MRP21* suppressor mutations

Relevant genotype	Growth with *MRP21* allele[a]:		
	MRP21	*MRP21-1*	*MRP21-3*
COX3, *COX2*	++++	++++	++++
cox3-15	±	+++	+++
pet54-A244, *cox3-15*	−	+	ND
cox3-438	−	−	−
cox3-1	+	±	++
cox2-11	±	++	++
cox2-105	±	+	++
cox2-12	−	−	−
cox2-10	±	±	+

[a] The number of + signs indicates relative level of respiratory growth, determined by measuring the colony size on YPEG medium at 30°C, except for *cox3-15* mutants, which were incubated at 13.5°C. −, no growth; ND, not determined. Suppression was tested in haploids, except with *cox2-12*, *cox2-105*, and *cox3-438*, for which dominant suppression in heterozygous diploids is reported. References for the alleles listed in the table are as follows: *cox3-15* (12); *pet54-A244* (6); *cox3-438* (67); *cox3-1* (26); *cox2-11* (45); *cox2-105* (22); *cox2-12* (45); *cox2-10* (47). The following alleles showed no genetic interaction (neither suppression nor synthetic defects) with *MRP21-1*: *cox3-13* and *-14* (67); *cox3-209* (13); *cox2-13* (45); *cox2-103*, *cox2-106*, *cox2-107*, and *cox2-108* (22); *MRP51-1*, *MRP51-2*, *MRP51-3*, *MRP51-4*, and *MRP51-5* (this study); *pet54-A244* (6); *PET122-L195* (12); *PET122-I175* and *PET122-V211* (6); *oli1-h45* (51).

One possible mechanism for suppression of 5′-UTL mutations in both the *COX2* and *COX3* mRNAs is that the alterations in Mrp21p and Mrp51p cause a general increase in the level of mitochondrial translation. To test this possibility, we asked whether the *MRP21* and *MRP51* suppressor alleles would improve the respiratory growth of strains with leaky initiation codon mutations (AUG to AUA) in these mitochondrial mRNAs (*cox2-10* and *cox3-1*). Since the respiratory growth of these mutants is responsive to levels of translational activity (26, 47), we would expect that all of the *MRP21* and *MRP51* suppressor alleles would suppress the initiation codon mutations if the mechanism of suppression were an overall increase in translation. We might also expect that the strength of suppression of 5′-UTL mutations would correlate with the strength of suppression of initiation codon mutations for each particular suppressor allele: a strong suppressor of 5′-UTL mutations would strongly suppress initiation codon mutations, and the converse. However, this was not the case. Some alleles that suppressed 5′-UTL mutations failed to affect the initiation codon mutants, others suppressed them, and still others reduced their respiratory growth, causing a synthetic defect (Tables 2 and 3).

In the experiment in Fig. 2, this phenomenon is illustrated at the level of mitochondrial protein synthesis for the *MRP51-3* allele. *MRP51-3* had a dramatic effect on the *cox2-11* 5′-UTL mutation: in the unsuppressed strain, the level of Cox2p was greatly reduced (Fig. 2, lane 3), while in an *MRP51-3 cox2-11* strain, Cox2p was synthesized at wild-type levels (lane 4). In a leaky *cox2* initiation codon mutant strain, Cox2p levels were reduced from wild-type levels, whether or not the strain carried *MRP51-3* (lanes 5 and 6). Similarly, *MRP51-3* had no effect on Cox3p synthesis from the leaky *cox3* initiation codon mutant allele (lanes 7 and 8). Levels of Cox2p and Cox3p synthesis in these strains correlated with their respiratory growth phenotypes, confirming that *MRP51-3* strongly suppressed the 5′-UTL mutation while having no effect on the initiation codon mutations.

In vivo labeling of mitochondrial translation products in

TABLE 3. Allele specificity of the *MRP51* suppressor mutations

Relevant genotype	Growth with *MRP51* allele[a]:					
	MRP51	*MRP51-1*	*MRP51-2*	*MRP51-3*	*MRP51-5*	*MRP51-8*
COX2	++++	++++	++++	++++	++++	++++
cox2-11	±	++	++	+++	++	−
cox2-12	−	−	−	++	±	−
cox2-13	−	−	−	−	−	−
cox2-10	+	++	+++	+	+++	±
cox2-105	±	+	+	+++	++	±
cox3-1	+	+	+	+	+	+
cox3-15	±	±	+	+	++	++
cox3-438	−	−	−	−	−	−

[a] The number of + signs indicates the relative level of respiratory growth, determined by measuring the colony size on YPEG medium at 30°C, except for *cox3-15* mutants, which were incubated at 13.5°C. −, no growth. Suppression was tested in haploids except with *cox2-105* and *cox3-438*, for which dominant suppression in heterozygous diploids is reported. References for the alleles listed in the table are indicated in the legend to Table 2. *MRP51* suppressors did not exhibit dominant suppression of the *cox2-103*, *cox2-106*, *cox2-107*, and *cox2-108* alleles (22) and the *oli1-h45* alleles (51).

MRP21-1 strains with *cox2* and *cox3* initiation codon mutations (not shown) yielded similar results. The *MRP21-1* allele, a strong suppressor of the 5′-UTL mutation *cox3-15*, had no effect on Cox2p synthesis in the *cox2* initiation codon mutant strain. In combination with the *cox3* initiation codon mutation, *MRP21-1* caused reduced Cox3p synthesis, consistent with the synthetic respiratory defect observed in the double-mutant strain (Table 2).

Molecular cloning and nucleotide sequence analysis of the *MRP21* and *MRP51* genes. The dominant suppression phenotypes of the *MRP21* and *MRP51* suppressors were used to clone both genes. A genomic library was constructed in a multicopy plasmid from a strain (MCC267; Table 1) carrying both the *MRP21-1* and *MRP51-3* alleles, to isolate both genes from a single library (Materials and Methods). To clone *MRP21*, the *cox3-15* mutant strain TF210 (Table 1) was transformed with the library and transformants with cold-resistant respiratory growth were selected. To clone *MRP51*, the *cox2-12* mutant strain JJM158 (Table 1) was transformed with the library and respiring transformants were selected.

Plasmids that conferred cold-resistant respiratory growth on the *cox3-15* mutant strain TF210 fell into two distinct sets based on restriction mapping and hybridization analysis. The nucleotide sequences of small fragments from each class were determined to localize the plasmid inserts in the genome. One class of plasmids was found to carry *PET494*, which encodes a subunit of the *COX3*-specific translational activator (9, 10) and is known to suppress *cox3-15* when overexpressed (12). The other class of plasmids conferring cold-resistant respiratory growth carried a region of DNA near the *ROX3* gene (57) from chromosome II. To test whether the *MRP21* gene was located in this region of the genome, the *URA3* gene was integrated into the *ROX3* region of a strain carrying the *cox3-15* allele and the integrant strain was crossed to an *MRP21-1* strain also carrying *cox3-15* (see Materials and Methods). Among the meiotic progeny of this cross, all the spores that were able to respire at 13.5°C were Ura^- whereas all Ura^+ spores had cold-sensitive respiratory growth: thus, the *MRP21* gene is tightly linked to the *ROX3* region.

A 2.9-kb *Bam*HI fragment which included 1.8 kb from the region common to all six plasmids obtained from the genomic library carried the complete *MRP21-1* gene, as judged by its ability when subcloned to confer cold-resistant respiration on the *cox3-15* strain TF210 as well as did the original library plasmids. This fragment carried two open reading frames, but only one was located entirely within the region of overlap of the library plasmids, identifying it as *MRP21*. The *MRP21* open reading frame (YBL090W; GenBank accession no. Z35851) encodes a strongly basic (net charge of +18) 177-amino-acid protein with a predicted molecular mass of 20.4 kDa. Homology searches with the Basic Local Alignment Search Tool (BLAST) program (2) revealed weak similarity between the

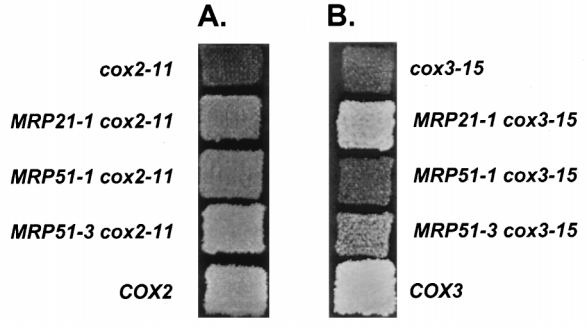

FIG. 1. Suppression of selected *cox2* and *cox3* 5′-UTL mutations by *MRP21* and *MRP51* mutations. Cells were grown on glucose-containing medium (YPD), printed to nonfermentable medium (YPEG medium supplemented with 0.02 mg of adenine per ml), and incubated at 30°C for 2 days (A) or at 13.5°C for 12 days (B). Relevant genotypes are shown. Where it is not indicated otherwise, *MRP21* and *MRP51* are wild type.

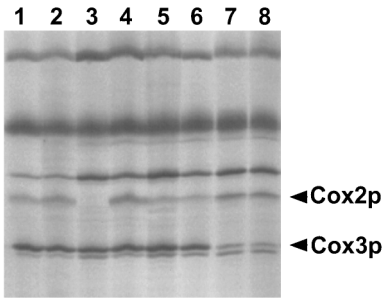

FIG. 2. Effects of the *MRP51-3* suppressor on Cox2p and Cox3p synthesis. Mitochondrial translation products were radioactively labeled in the presence of cycloheximide and subjected to electrophoresis as described in Materials and Methods. The positions of Cox2p and Cox3p are indicated. Strain names and relevant genotypes are as follows: lane 1, DL1 (wild-type); lane 2, NSG78 (*MRP51-3*); lane 3, JJM120 (5′-UTL mutation *cox2-11*); lane 4, NSG50 (*MRP51-3 cox2-11*); lane 5, JJM113 (initiation codon mutation *cox2-10*); lane 6, NSG59 (*MRP51-3 cox2-10*); lane 7, LSF75 (initiation codon mutation *cox3-1*); lane 8, NSG83 (*MRP51-3 cox3-1*).

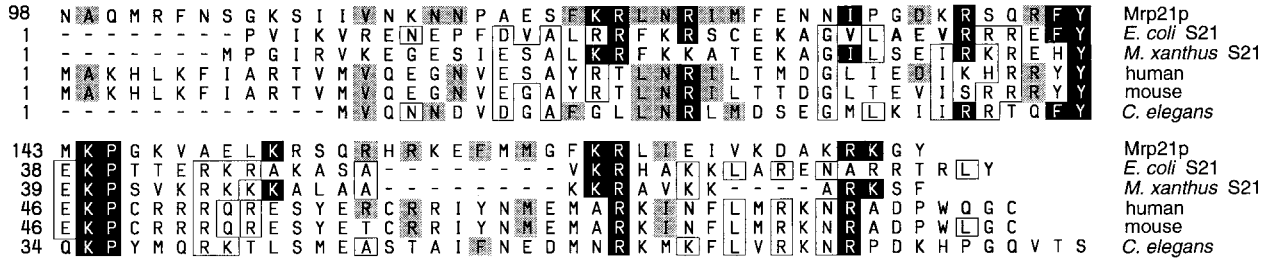

FIG. 3. Alignment of the C-terminal region of Mrp21p with bacterial ribosomal S21 proteins and with sequences from higher eukaryotes. Black boxes, identities between Mrp21p and either or both of the bacterial S21 proteins; gray boxes, identities between Mrp21p and the metazoan proteins not shared by the bacterial S21 proteins; white boxes, identities between the metazoan proteins and the bacterial S21 proteins not found in Mrp21p. The percentages of identical plus similar amino acids for selected pairwise comparisons are as follows: Mrp21p-*E. coli* S21, 23.9%; Mrp21p-*Myxococcus xanthus* S21, 28.1%; Mrp21p-human, 36.1%; Mrp21p-mouse, 32.5%; Mrp21p-*C. elegans*, 25.3%; human-*E. coli* S21, 34.3%; human-*M. xanthus* S21, 39.1%. The accession numbers of the sequences are as follows: Mrp21p, Z35851; *E. coli rpsU* gene, V00346 (40); *M. xanthus rpsU* gene, U20669; human, coordinates 447 to 710, U79258; mouse, coordinates 73 to 336, AA050698; *C. elegans* gene F29B9.10, U70849.

C-terminal region of Mrp21p and predicted proteins of unknown function from humans, mice, and *Caenorhabditis elegans*, all of which are highly homologous. The metazoan proteins exhibit a clear similarity to the small-subunit ribosomal S21 protein from bacteria. Alignment of the Mrp21p, higher eukaryotic, and bacterial sequences (Fig. 3) reveals clear similarities between Mrp21p and S21. Interestingly, the sequences of the suppressor alleles (see Materials and Methods) revealed that they caused missense substitutions in a small region of the S21-homologous C-terminal domain of Mrp21p. The independently isolated *MRP21-1* and *MRP21-2* alleles were identical (see Materials and Methods), both causing a Glu-to-Lys change at amino acid 118. The *MRP21-3* allele changed Asn to Lys at amino acid 124, increasing the similarity to bacterial S21 proteins.

To clone *MRP51*, the *cox2-12* mutant strain JJM158 was transformed with the MCC267 library and respiring transformants were selected. Fourteen overlapping plasmids which conferred respiratory growth were isolated. To test whether *MRP51* was linked to this region, a fragment from this plasmid was cloned into an integrating vector carrying the *URA3* gene and transformed into a *MRP51-5*/*MRP51* diploid (see Materials and Methods). When independent diploid transformants were sporulated and tetrads were dissected, the ability to respire segregated either with the Ura^+ marker (integration into the suppressor chromosome) or opposite to the Ura^+ marker (integration into the wild-type chromosome). Thus, the plasmid-borne sequences were tightly linked to *MRP51.*

Sequence analysis of the smallest library plasmid revealed two complete open reading frames: the *IDI1* gene encoding isopentenyl diphosphate-dimethylallyl diphosphate isomerase, an enzyme of the isoprenoid biosynthetic pathway (3), and an unidentified gene. To determine whether the unidentified open reading frame was *MRP51*, it was subcloned from the suppressing plasmid after PCR amplification (Materials and Methods). The resulting plasmid, pNSG19, had suppressor activity in *cox2-11* and *cox2-12* mutant strains, identifying this gene as *MRP51*. The *MRP51* open reading frame (YPL118W; coordinates 16771 to 17805 of GenBank sequence no. U43503) encodes a 344-amino-acid protein with a predicted molecular mass of 39.5 kDa. Mrp51p is predicted to be a strongly basic protein with a net charge of +22. The DNA sequences of the suppressor alleles (see Materials and Methods) revealed that they were associated with amino acid substitutions in a limited region of Mrp51p: *MRP51-1*, Val to Ala at position 235; *MRP51-2*, Asp to His at position 241; *MRP51-3* and *MRP51-4*, Pro to Arg at position 261; *MRP51-5*, Pro to Leu at position 260; *MRP51-8*, Glu to Arg at position 279. No proteins of known function are homologous to Mrp51p. However, Mrp51p is 46% identical to an unidentified open reading frame of *S. kluyveri* (66) (coordinates 290 to 378 of GenBank sequence no. U83662 and coordinates 2569 to 1543 of EMBL sequence no. Z14125).

Construction and characterization of *mrp21* and *mrp51* null mutations. To inactivate *MRP21* and *MRP51*, internal fragments of both genes were removed and replaced with a *hisG*::*URA3*::*hisG* cassette (1) (see Materials and Methods). DNA fragments carrying each deleted and disrupted gene were used, separately, to transform a diploid strain homozygous for a *ura3* mutation. In each case, the Ura^+ diploid transformants respired well, but when the diploids were sporulated and tetrads were dissected, each tetrad had two respiratory-competent Ura^- spores and two respiratory-deficient Ura^+ spores. The Ura^+ spores were unable to produce respiring diploids when mated to a nuclearly wild-type, rho^0 tester strain (lacking mitochondrial DNA), indicating that deletion of either *MRP21* or *MRP51* caused the cells to lose their mitochondrial DNA. The destabilization of mitochondrial DNA is a hallmark of mutations that block all mitochondrial translation (24, 34, 49, 50). This suggested that both Mrp21p and Mrp51p might be required generally for mitochondrial translation.

Subcellular and submitochondrial localization of Mrp21p and Mrp51p. To detect Mrp21p, it was tagged at the carboxy terminus with three copies of the influenza virus HA epitope (25, 65) (see Materials and Methods), which is recognized by the 12CA5 mouse monoclonal antibody. HA-tagged Mrp21p was only partially functional: strains in which the only copy of *MRP21* carried the HA tag showed a mild respiratory defect. To detect Mrp51p, we raised a rabbit polyclonal antiserum to a version of Mrp51p carrying amino- and carboxy-terminal six-histidine tags (37), purified after expression in *E. coli* (see Materials and Methods).

Wild-type yeast (PTY11) and a strain carrying a chromosomally integrated gene encoding HA-tagged Mrp21p (MCC291) were grown and fractionated into mitochondrial pellets and postmitochondrial supernatant fractions, after which the mitochondria were purified by buoyant density gradient centrifugation (see Materials and Methods). The fractions were analyzed by gel electrophoresis and Western blotting, probing either with the anti-HA monoclonal antibody or with the polyclonal anti-Mrp51p antiserum (Fig. 4). As expected, both Mrp21p and Mrp51p were associated specifically with mitochondria.

The phenotypes of the *mrp21* and *mrp51* null mutations

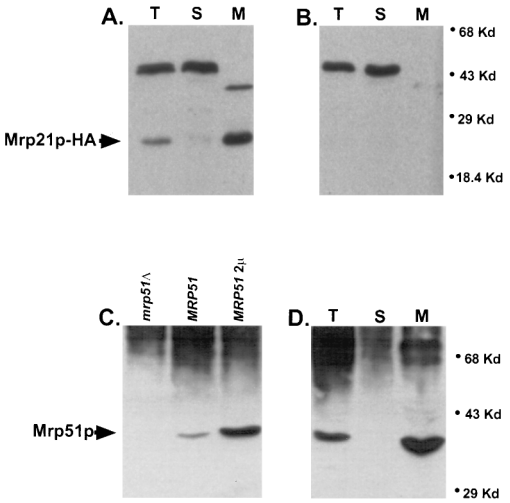

FIG. 4. Mrp21p and Mrp51p are located in mitochondria. (A and B) Subcellular location of Mrp21p-HA (arrow). A 50-μg portion of protein was applied to each lane of the gel. Western blots in both panels were probed with monoclonal anti-HA antibody. (A) Subcellular fractions of an *MRP21-HA* strain (MCC291). Mrp21p-HA is present in whole-cell extract (lane T) and gradient-purified mitochondria (lane M) but not in the cytosol (lane S). (B) Corresponding fractions from a wild-type strain (PTY11). (C) The polyclonal anti-Mrp51p antibody detects an approximately 39-kDa protein in whole-cell extract from the wild type (*MRP51*; strain DAU1) that is absent in a null mutant (*mrp51*Δ; NSG63) and overproduced in a strain carrying *MRP51* on a high-copy-number plasmid (*MRP51* 2μm; plasmid pNSG22 in strain DAU1), identifying this band as Mrp51p. Approximately 10 μg of total-cell protein was applied per lane. (D) Subcellular location of Mrp51p (arrow). The anti-Mrp51p antibody detects Mrp51p in whole-cell extract (lane T) and gradient-purified mitochondria (lane M) but not in the cytosol (lane S) of a wild-type strain (PTY11). The amounts of protein applied to the gel were 50 μg (lane T), 20 μg (lane S), and 20 μg (lane M).

suggested that they were required for all mitochondrial translation. This, as well as the similarity between Mrp21p and bacterial S21 proteins, raised the possibility that they were components of the mitochondrial ribosome. To test this possibility, gradient-purified mitochondria were solubilized with detergent and the contents were sedimented into a sucrose gradient in the presence of high salt concentrations (0.5 M NH_4Cl; see Materials and Methods) to separate the subunits of mitochondrial ribosomes. The gradient fractions were analyzed for absorbance at 260 nm, to locate the rRNAs, and by gel electrophoresis and Western blotting (Fig. 5 and 6). In the experiment in Fig. 5, mitochondrial ribosomes from wild-type (PTY11) cells were subjected to this analysis. The position of Mrp51p coincided with that of the ribosomal small subunit, as identified by the smaller of two peaks of absorbance at 260 nm and by the presence of Mrp13p, a known small-subunit constituent (53). In the experiment in Fig. 6, a similar analysis was performed on mitochondrial ribosomes isolated from a strain (MCC291) in which the only functional *MRP21* gene carried the HA tag at its 3′ end. As noted above, the HA-tagged Mrp21p did not function as well as the wild type, and the experiment in Fig. 6 reveals the probable reason for this. The small subunit of mitochondrial ribosomes was partially destabilized in this strain, as shown by the dramatic decrease in the peak of absorbance for the small rRNA relative to that for the large rRNA. Nevertheless, the peak of Mrp21p-HA coincided with the small ribosomal subunit. Indeed, the fact that an alteration which decreased the function of Mrp21p specifically affected the small subunit of mitochondrial ribosomes strongly supports the idea that Mrp21p is a small-subunit component.

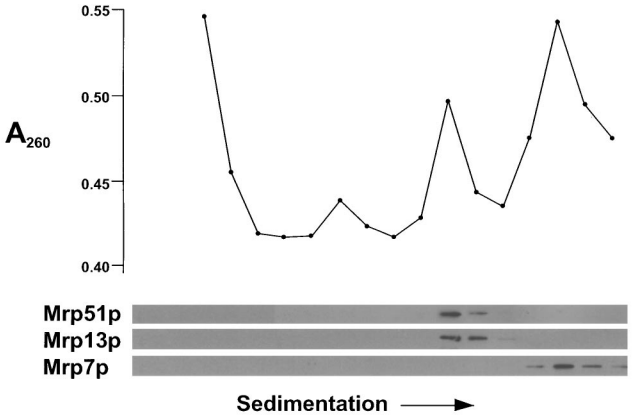

FIG. 5. Mrp51p cosediments with the small subunit of mitochondrial ribosomes. Nycodenz gradient-purified mitochondria of strain PTY11 were disrupted with deoxycholate, and the soluble contents were centrifuged into a sucrose gradient in the presence of 0.5 M salt (see Materials and Methods). (Top) Absorbance at 260 nm (A_{260}) of alternate fractions. (Bottom) Western blots of alternate fractions probed with antisera against Mrp51p, the known small-subunit protein Mrp13p (53), and the known large-subunit protein Mrp7p (24).

DISCUSSION

We have identified two nuclear yeast genes encoding previously unidentified mitochondrial ribosomal small-subunit proteins, Mrp21p and Mrp51p. These genes can mutate to suppress defects in the 5′-UTLs of two different mitochondrial mRNAs, *COX2* and *COX3*, but do not bypass the mRNA-specific translational activation system. The 5′-UTL mutations are known from previous genetic analysis to alter the targets of the *COX2* and *COX3* mRNA-specific translational activators (12, 46). However, the functions of the *MRP21* and *MRP51* products are not mRNA specific. Suppressor alleles at each of these two nuclear genes were able to improve the respiratory growth of certain 5′-UTL mutations, but not others, affecting both the *COX2* and *COX3* mRNAs. Furthermore, deletion of either *MRP21* or *MRP51* prevented mitochondrial translation globally.

Suppression of 5′-UTL mutations might occur by any alteration that caused a general increase in mitochondrial transla-

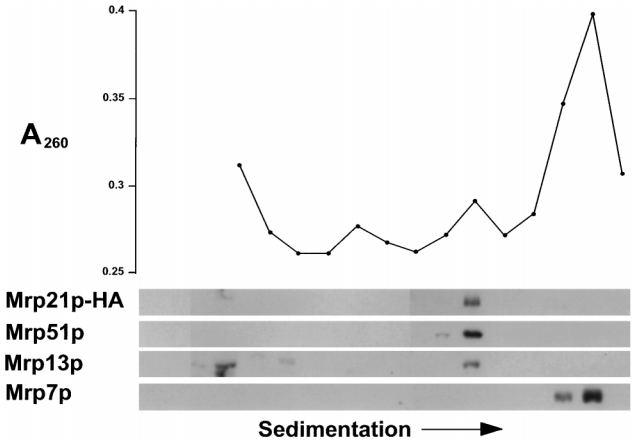

FIG. 6. Partially functional Mrp21p-HA cosediments with the small subunit of mitochondrial ribosomes but destabilizes it. Nycodenz gradient-purified mitochondria of strain MCC291 were analyzed as described in the legend to Fig. 5, except that the fractions were also probed with the anti-HA monoclonal antibody.

tional activity. However, this does not appear to be the mechanism by which the *MRP21* and *MRP51* suppressors work, since many of the suppressors failed to increase the respiratory growth of strains bearing leaky *COX2* and *COX3* initiation codon mutations (*cox2-10* and *cox3-1*) and also failed to increase Cox2p or Cox3p synthesis in these strains. The initiation codon mutations reduce the translation of mRNAs bearing otherwise wild-type 5′-UTLs roughly five- to sevenfold without altering the sites of initiation (26, 47). Furthermore, the growth phenotypes they cause are influenced by the levels of their respective mRNA-specific translational activators, indicating that they are sensitive to translational activity (26, 47). While some of the other suppressor alleles did improve the growth of initiation codon mutants, *MRP21-1* and *MRP51-8* actually reduced the respiratory growth of *cox3-1* and *cox2-10* mutants, respectively. Thus, we conclude that the *MRP21* and *MRP51* mutations do not generally increase the activity of mitochondrial ribosomes. Instead, the patterns of suppression by the *MRP21* and *MRP51* mutations, which are allele specific but, surprisingly, gene nonspecific, suggest that yeast mitochondrial ribosomes may recognize a common feature in mRNA 5′-UTLs. According to this hypothesis, the structure of the common element was altered by mutations in the *COX2* and *COX3* 5′-UTLs and the suppressors altered the ribosomal small subunit to compensate for the defects.

Mrp21p resembles several other yeast mitochondrial small-subunit ribosomal proteins (4, 18, 19, 39) in that it has a domain lacking homology to any known protein and a domain identifiably homologous to a bacterial ribosomal protein. The amino-terminal 99-amino-acid sequence of Mrp21p is not similar to currently known sequences, but the carboxy-terminal 78-residue sequence exhibits clear similarity to a metazoan sequence, which in turn is clearly similar to those of bacterial ribosomal S21 proteins. This family of proteins is absent in eukaryotic cytoplasmic ribosomes (68) and those of known members of the *Archaea* (7). The limited homology is convincing when taken together with the facts that both Mrp21p and S21 are small-subunit ribosomal proteins and that our suppressors are missense substitutions in a small region of the S21-homologous C-terminal domain of Mrp21p. The functions of the metazoan Mrp21p homologs are unknown, but it is likely that they are also mitochondrial ribosomal proteins involved in translation initiation.

The available evidence is consistent with the idea that Mrp21p and bacterial S21 may have similar functions in promoting mRNA-ribosome interactions. Our genetic data suggest that Mrp21p may interact directly with the 5′-UTLs. Ribosomal protein-mapping studies indicate that *E. coli* S21 protein is in the platform region of the small subunit, the site of Shine-Dalgarno and codon-anticodon interactions (8). S21 is in very close proximity to both the 16S rRNA and the initiation region of mRNAs, as shown by cross-linking and resonance energy transfer experiments (15, 21, 48). However, the in vivo function of S21 has not been studied genetically. The only reported alleles of the *E. coli* gene encoding S21, *rpsU*, have no effect on translation (16).

Like several other mitochondrial ribosomal small-subunit proteins (33, 42, 49, 53), Mrp51p exhibits no clear homology to any known ribosomal proteins. The only known homolog is the product of an unidentified open reading frame in the yeast *S. kluyveri*, which probably also encodes a mitochondrial ribosomal protein. Interestingly, the missense substitutions caused by our five different *MRP51* suppressor alleles are clustered within a 45-amino-acid region of the protein, which could be involved in mRNA interactions.

The mechanism by which yeast (and other) mitochondrial ribosomes identify translation initiation sites is not clear, largely owing to the lack of suitable in vitro systems (20). However, it does not involve either a classical Shine-Dalgarno interaction or a simple scanning mechanism (27). AUG codons clearly play a role in start site selection, but additional information is also used (26, 47). Genetic studies have strongly supported a model in which mRNA-specific translational activators mediate the mRNA-ribosome interaction leading to initiation and possibly influence start site selection (reviewed in reference 27).

Our present data demonstrate that, in conjunction with mRNA-specific activators, the yeast mitochondrial ribosome itself plays an active role in recognizing translatable mRNAs. The pattern of suppression observed suggests that the ribosomes may recognize a feature common to all yeast mitochondrial mRNA 5′-UTLs. A candidate for such a common feature, the octanucleotide sequence UAUAAAUA, has recently been identified based on a functional analysis of the *COX2* mRNA 5′-UTL and comparisons with other 5′-UTLs (22). While this sequence is not directly altered in the suppressible alleles studied here, *cox2-11* and *cox3-15*, it is within 10 bases upstream of both mutations. This octanucleotide is complementary to several sites in the mitochondrial small-subunit rRNA and could thus be involved in mRNA-rRNA base pairing. Clearly, Mrp21p and Mrp51p could play a role in establishing such an mRNA-rRNA interaction. However, this putative interaction would not closely resemble the Shine-Dalgarno mechanism (55, 61), since the octanucleotide does not occur at fixed distances from translation initiation codons of mRNAs and its complement is not located at the 3′ end of the rRNA. Alternatively, yeast mitochondrial ribosomes could interact with mRNAs purely through protein-mRNA contacts, possibly involving Mrp21p and Mrp51p directly.

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