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In Vivo Analysis of Sequences Required for Translation of Cytochrome *b* Transcripts in Yeast Mitochondria

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Respiratory chain proteins encoded by the yeast mitochondrial genome are synthesized within the organelle. Mitochondrial mRNAs lack a 5′ cap structure and contain long AU-rich 5′ untranslated regions (UTRs) with many potential translational start sites and no apparent Shine-Dalgarno-like complementarity to the 15S mitochondrial rRNA. However, translation initiation requires specific interactions between the 5′ UTRs of the mRNAs, mRNA-specific activators, and the ribosomes. In an initial step toward identifying potential binding sites for the mRNA-specific translational activators and the ribosomes, we have analyzed the effects of deletions in the 5′ UTR of the mitochondrial *COB* gene on translation of *COB* transcripts in vivo. The deletions define two regions of the *COB* 5′ UTR that are important for translation and indicate that sequence just 5′ of the AUG is involved in selection of the correct start codon. Taken together, the data implicate specific regions of the 5′ UTR of *COB* mRNA as possible targets for the mitochondrial translational machinery.

In *Saccharomyces cerevisiae*, seven components of the respiratory chain complexes and the ATP synthase are encoded by mitochondrial genes and synthesized by mitochondrial ribosomes (reviewed in references 18, 26, 69, and 71). While mitochondrial ribosomes and general translation factors are similar to their prokaryotic counterparts (27, 37, 43, 53, 54, 72), many features of mitochondrial translation appear to be quite different from translation in either prokaryotic cells or the cytoplasm of eukaryotic cells.

Of particular interest is the mechanism by which mitochondrial ribosomes recognize mitochondrial mRNAs and select the correct start codon to initiate protein synthesis. Mitochondrial mRNAs lack a 5′ cap structure (6) and contain long AU-rich 5′ untranslated regions (UTRs) with many potential start codons; therefore, it is generally believed that mitochondrial ribosomes do not utilize a scanning mechanism similar to that proposed for cytoplasmic ribosomes (38). Prokaryotic mRNAs show sequence conservation from positions −20 through +13 (all positions are given relative to the AUG at +1), including the Shine-Dalgarno sequence complementary to the 3′ end of the 16S rRNA (reviewed in references 24 and 28). Similar potential ribosome-binding sites within the 5′ UTRs of mitochondrial mRNAs are not immediately apparent. Sequences complementary to the 3′ end of the mitochondrial 15S rRNA have been identified (42), but the extent of complementarity and distance from the start codon vary between mitochondrial mRNAs. Whether these sequences play a role similar to that of the Shine-Dalgarno sequence in prokaryotic mRNAs is not known. Other than the high A+U content (95%), the only striking feature common to the 5′ UTRs of mitochondrial mRNAs is the presence of an AUG start codon. However, when the AUG start codon in either *COX2* or *COX3* mRNA (encoding cytochrome oxidase subunits II and III, respectively) was changed to AUA, translation was reduced but not eliminated (21, 51). Therefore, as for both prokaryotic (24) and eukaryotic (38) mRNAs, the AUG start codon is not the only sequence element involved in start site selection.

Another intriguing feature of translation initiation in yeast mitochondria is the requirement for translational activators that are encoded by nuclear genes and stimulate translation of specific mitochondrial mRNAs (reviewed in references 8 and 18). For example, translation of mitochondrial *COB* mRNA, encoding cytochrome *b*, is dependent on the nuclear *CBS1* and *CBS2* gene products (58, 61) whereas translation of *COX3* mRNA is dependent on the products of three nuclear genes: *PET54*, *PET494*, and *PET122* (10–12). Biochemical analyses have shown that these mRNA-specific translational activator proteins are associated with the inner mitochondrial membrane (46–48), while suppressor analyses indicate that they act via the 5′ UTRs of the target mRNAs (7, 61, 62). Genetic data are suggestive of direct physical interactions between Pet122p and the *COX3* 5′ UTR (9) and between Pet122p and the small subunit of the mitochondrial ribosome (30, 31). In addition, a recent study points toward direct physical interactions between Pet54p and Pet122p and between Pet54p and Pet494p (4). These data have prompted a model in which a complex containing the mRNA-specific factors activates translation by mediating the interaction between mitochondrial mRNAs and ribosomes at the inner membrane (4, 47).

The 5′ UTR of the mature *COB* mRNA is 954 nucleotides in length and, as for other mitochondrial mRNAs, has no obvious features that suggest where the mitochondrial ribosomes, Cbs1p, and/or Cbs2p might bind. Therefore, in an attempt to define regions of the *COB* 5′ UTR that may interact with the translational apparatus, we have analyzed the effects of deletions within the 5′ UTR on *COB* expression. The data suggest that sequence elements between −232 and −4 are important for translation of *COB* transcripts and that the sequence between −33 and −4 is required for selection of the correct start codon. Also, we show that the nucleotides previously observed to be complementary to the 3′ end of the 15S rRNA are not required for *COB* expression.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains used in this study are listed in Table 1. Strains N356/*rho*^{0} and E67/*rho*^{0} were isolated following ethidium bromide mutagenesis of strains N356 and E67, respectively. Strains LL20/KL14 and N356/KL14 were constructed by transferring mitochondrial DNA from strain KL14-4B

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TABLE 1. Names and genotypes of yeast strains used in this study

Strain	Genotype	Reference
LL20/*rho*^{0}	α [*rho*^{0}] *leu2-3 leu2-112 his3-11 his3-15* 2μm$^+$	45
D273-10B/A21	α [*rho*$^{+A21}$(short)] *met6* E^r O^r P^r	68
A21	[*rho*$^{+A21}$(short)] derivative of LL20	50
CP1L/*rho*^{0}	*LEU2* insertion in *CBP1* in strain LL20	45
KL14-4B	α [*rho*$^{+KL14}$(long)] *his*	74
LL20/KL14	[*rho*$^{+KL14}$(long)] derivative of LL20	This study
N356	α [*rho*$^+$(short)] *met6 cbs1*	69
N356/*rho*^{0}	[*rho*^{0}] derivative of N356	This study
N356/KL14	[*rho*$^{+KL14}$(long)] derivative of N356	This study
E67	α [*rho*$^+$(short)] *met6 cbs2*	52
E67/*rho*^{0}	[*rho*^{0}] derivative of E67	This study
aM17-162-4A	**a** [*rho*$^+$, *mit*$^-$] *ade1*	70
M9410	α [*rho*$^{+M9410}$(long), *mit*$^-$] *ade1 op1*	19
JC3/*rho*^{0}	**a** [*rho*^{0}] *kar1-1 ade2 lys2*	1
JC3/M9410	[*rho*$^{+M9410}$(long), *mit*$^{-rho^0}$	50
W303Δcbp7/*rho*^{0}	α [*rho*^{0}] *ade2-1 his3-11 his3-15 leu2-3 leu2-112 ura3-1 trp1-1 can1-100 CBP7*::*URA3*	52
682L/282R	[*rho*$^{+682L/282R}$(long)] derivative of LL20	This study
682L/170R	[*rho*$^{+682L/170R}$(long)] derivative of LL20	This study
682L/4R	[*rho*$^{+682L/4R}$(long), *mit*$^-$] derivative of LL20	This study
JC3/682L-4R	[*rho*$^{+682L/4R}$(long), *mit*$^-$] derivative of JC3	This study
104L/4R	[*rho*$^{+104L/4R}$(long), *mit*$^-$] derivative of LL20	This study
33L/4R	[*rho*$^{+33L/4R}$(long), *mit*$^-$] derivative of LL20	This study
CP1L/33L-4R	[*rho*$^{+33L/4R}$(long), *mit*$^-$] derivative of CP1L	This study
104L/60R	[*rho*$^{+104L/60R}$(long)] derivative of LL20	This study
232L/60R-1	[*rho*$^{+232L/60R}$(short)] derivative of LL20	This study
232L/60R-2	[*rho*$^{+232L/60R}$(long)] derivative of LL20	This study
TG955 898L/282R	[*rho*$^{+TG955\ 898L/282R}$(short)] derivative of LL20	This study
MSD2-1	[*rho*$^{+MSD2}$] derivative of LL20	This study
MSD2-4	[*rho*$^{+MSD2}$] derivative of LL20	This study
mp707A	[*rho*$^{-707A}$] derivative of LL20	50

to strains LL20/*rho*^{0} and N356/*rho*^{0} by cytoduction via the *kar1* strain JC3/*rho*^{0} (40). Strain CP1L/33L-4R was constructed by transferring mitochondrial DNA from strain 33L/4R to strain CP1L/*rho*^{0} by cytoduction via the *kar1* strain JC3/*rho*^{0}.

We used YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and WO (0.67% yeast nitrogen base without amino acids, 2% glucose) as media. Solid media contained 2% agar.

Construction of deletions within the *COB* 5′ UTR. Plasmids Mb247/6-74 and Mb247/5-57 contain *COB* sequence from −1350 to +1716 ligated into the *Bam*HI site of the *Escherichia coli* plasmid pBluescript (Stratagene, La Jolla, Calif.) in the 5′-to-3′ and 3′-to-5′ orientations, respectively (50). Plasmids pJ6-41 and pΔ627 were obtained by exonuclease III plus mung bean nuclease treatment of Mb247/6-74 (50) and contain *COB* sequence from −1350 to −682 and from −1350 to −232, respectively. In these plasmids, an *Eco*RI site is located just 3′ of the *COB* sequence. Plasmids pL5-11 and pL5-34 were obtained by exonuclease III plus mung bean nuclease treatment of Mb247/5-57 (50) and contain *COB* sequence from −282 or −170 through +1716, respectively. In these plasmids, *Eco*RI sites are located at +654 of *COB* and, in pL5-11, just 5′ of the *COB* sequence. To construct plasmid p682L/282R, the *Eco*RI fragment from pL5-11 was ligated into *Eco*RI-digested pJ6-41 and plasmid p682L/170R was constructed by ligating the −170 to +1495 *Cla*I fragment from pL5-34 into *Cla*I-digested pJ6-41. To construct plasmid pTG955 898L/282R, *COB* sequence from −1350 to −898 from strain TG955 (5), in which sequence from −1098 to −955 is deleted, was amplified by PCR (with primers cob1714 and cob1718; all oligonucleotide primers are listed in Table 2) and the PCR product was ligated into *Bam*HI-*Eco*RI-digested pBluescript, yielding pKS/TG955-tRNA. The −282 to +654 *Eco*RI fragment from pL5-11 was then ligated into *Eco*RI-digested pKS/TG955-tRNA.

Plasmid p682L/4R was constructed by PCR amplification of *COB* sequence from −4 to +654 (primers cobATG and cob654A) and the *Eco*RI-digested PCR product was ligated into *Eco*RI-digested pJ6-41. To construct plasmids p104L/4R and p33L/4R, *COB* sequence from −1350 to −104 and from −1350 to −33 was amplified by PCR (primers cob1714 plus cob7B and cob1714 plus cob8B, re-

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence[a]	*COB* sequence
cob1714	AGGAGTGAT**GGATCC**CTTTGG	−1360 to −1341
cob1717	**CCGAATTC**ATAATAATAAATACC	−961 to −943
cob4083	**GGGAATTC**GAGGTTTATATTTAATAAATAAT	−324 to −301
cob3463	**CCGAATTC**ATATATAAAATATTAGTAATAAA	−60 to −38
cobATG	**GGGAATTC**TAATATGGCATTTAG	−4 to +11
cob1718	**CCGAATTC**TAATGAAAAATATATTATATA	−898 to −918
cob7B	**CCGAATTC**CTTATTATATATATATATATTT	−104 to −125
MSD2	ATATGAATTC**G*A*A*T***TTATATATATATA	−57 to −120[b]
cob8B	**CCGAATTC**TATTATTTATTACTAATATTTT	−33 to −54
cob4082	**CCGAATTC**TAATGAACCCATATTTCATCAA	+105 to +84
cob654A	GAATGCATTG**GAATTC**TATC	+668 to +649[c]

[a] The primer sequence is in the 5′-to-3′ orientation. Restriction endonuclease recognition sequences have been underlined. Sequence that is not identical to that of *COB* is in boldface type.

[b] The MSD2 primer sequence is identical to that in the 104L/60R construct except for nucleotides −104 through −107, which are marked by asterisks.

[c] Equivalent to +4172 to +4153 of the long form of *COB*.

spectively) and the *Bam*HI-*Eco*RI-digested PCR products were used to replace the −1350 to −682 *Bam*HI-*Eco*RI fragment of p682L/4R.

To construct plasmid p232L/60R, *COB* sequence from −60 to +654 was amplified by PCR (primers cob3463 and cob654A) and the *Eco*RI-digested PCR product was ligated into *Eco*RI-digested pΔ627. Similarly, plasmid p104L/60R was constructed by replacing the −4 to +654 *Eco*RI fragment of p104L/4R with the *Eco*RI-digested −60 to +654 PCR product. Plasmid pMSD2 was obtained by PCR amplification of *COB* sequence from −1350 to −104 (primers cob1714 and MSD2, in which nucleotides −104 through −107 differ from the wild-type sequence), and the mutant PCR product was used to replace the wild-type −1350 to −104 *Eco*RI fragment of p104L/60R. In each construct, the deleted sequence was replaced with an *Eco*RI site, with the exception of deletion 682L/170R, in which the deleted sequence was replaced with a 24-bp linker sequence that included an *Eco*RI site.

Transformation of mitochondria by microprojectile bombardment. Yeast strain LL20/*rho*^{0} (α *leu2-3 leu2-112 his3-11 his3-15* [*rho*^{0}]) was cotransformed with YEp351 (a multicopy plasmid carrying the *LEU2* gene [33]) and each of the *COB* deletion plasmids by high-velocity microprojectile bombardment (36, 64) as described previously (50). Mitochondrial transformants were identified by their ability to rescue the *mit*$^-$ mutation in strain aM17-162-4A, which lies between +74 and +504 of *COB* (55, 56). Mitochondrial transformants obtained by this method have been termed synthetic *rho*$^-$ (petite) strains (23), because the plasmid DNA behaves similarly to a stably maintained petite mitochondrial genome. Transformation frequencies were similar to those previously reported (5, 22, 50).

Strain construction. The *COB* deletions in the synthetic *rho*$^-$ strains were introduced into otherwise wild-type mitochondrial genomes via recombination. Strains carrying the desired recombinant mitochondrial genomes were isolated following mating of each synthetic *rho*$^-$ strain to a karyogamy-deficient strain that was unable to respire because of a large deletion in the *COB* 5′ UTR (the mating procedure is given in reference 50). Strains mp682L/282R, mp682L/170R, mp682L/4R, mp232L/60R, and mp104L/60R (where mp stands for mitochondrial petite) were mated to strain JC3/M9410 (Table 1), while strains mp33L/4R, mp104L/4R, mpTG955 898L/282R, mpMSD2-1, and mpMSD2-4 were mated to strain JC3/682L-4R (Table 1). Depending on the point of recombination between the synthetic *rho*$^-$ genome, which was derived from a short-form *COB* gene (lacking introns bi1, bi2, and bi3), and the M9410 or 682L/4R mitochondrial genomes, which were long form (containing introns bi1, bi2, and bi3), the resulting mitochondrial genomes with deletions in *COB* contained either the long or the short form of *COB* (see Fig. 1). Cytoductants that carried the recombinant *COB* deletion genomes were identified by their ability to form respiratory competent diploids when mated to the synthetic *rho*$^-$ strain mp707A (50), which rescued the deletions in the desired *COB* deletion genomes but not those in the M9410 or 682L/4R mitochondrial genomes (strain 682L/4R was initially identified by rescue of another synthetic *rho*$^-$ strain, mp923A [50]).

Northern blot analyses. Isolation of mitochondrial RNA and preparation of the Northern (RNA) blots were performed as described previously (50). Northern blots were then prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (44)–1% Denhardt's solution (44)–0.1% sodium dodecyl sulfate (SDS) for 1 h at 42°C. ^{32}P-labeled oligonucleotide cob654A (Table 2; 5′ end labeled with T4 polynucleotide kinase [Promega, Madison, Wis.]) was added and hybridized for 20 h at 42°C. The blots were washed in 6× SSC–0.1% SDS twice for 10 min at 25°C and once for 20 min at 46°C and then exposed to X-ray film. The blots were stripped of probe in 1% SDS at 90 to 100°C, hybridized to an *OLI1*-specific probe, stripped again, and hybridized to a *COX1*-specific probe. The *OLI1*-specific and *COX1*-specific ^{32}P-labeled probes were obtained by random-primed synthesis of ^{32}P-labeled DNA with *OLI1* sequence from +179 to +797 or *COX1* sequence from plasmid EHox3 (67) as templates. Conditions used to analyze the Northern blots with the random-primed probes have been described previously (50). A separate blot was hybridized to a *COB* bi4-specific probe, which was obtained by random-primed synthesis of ^{32}P-labeled DNA with a 613-bp *Dra*I fragment derived from *COB* intron bi4 as a template. The signals obtained from *COB* or *COX1* transcripts were quantitated with a Betascope (Betagen, Waltham, Mass.) and were normalized to the signal obtained from *OLI1* transcripts in the same sample. The percentages given in Table 3 are averages of the values obtained from two separate Northern blots.

PCR amplification and sequencing of the deletion junctions in *COB* deletion strains. Reaction conditions for all PCR amplifications described in this study were as recommended by the supplier of *Taq*I polymerase (Promega, Madison, Wis., or Perkin-Elmer Cetus, Norwalk, Conn.). Mitochondrial DNA was isolated from the *COB* deletion strains as described previously (50). Primers cob1717 and cob7B (Table 2) were used to amplify sequence surrounding the deletion junctions in strains 682L/282R and 682L/170R. Primers cob4082 and cob4083 were used to amplify sequence surrounding the deletion junctions in strains 33L/4R, 104L/4R, 104L/60R, and 232L/60R. The *Eco*RI-digested PCR products were ligated into *Eco*RI-digested pBluescript. Similarly, primers cob1714 and cob7B (Table 2) were used to amplify sequence from −1350 to −104 in strain TG955 898L/282R and the *Bam*HI-*Eco*RI-digested PCR product was ligated into *Bam*HI-*Eco*RI- or *Eco*RI-digested pBluescript. After transformation of *E. coli* RR1 with the ligation mix, plasmid DNA from individual clones was isolated and the inserts were sequenced with Sequenase version 2.0 and the universal T3 or T7 primers (Stratagene) as recommended by the supplier of the enzyme (United States Biochemical Corporation, Cleveland, Ohio). In this manner, the following sequence surrounding the deletion junctions in each of the *COB* deletion strains was verified: strain 682L/282R from −781 to −104, strain 682L/170R from −855 to −126, strain 232L/60R-1 from −300 to +84, strain 104L/60R from −300 to −170 and from the *Eco*RI site at the deletion junction to +84, strain 104L/4R from −300 to +84, strain 33L/4R from −300 to +84, and strain TG955 898L/282R from −1100 to the *Eco*RI site at the deletion junction (−898).

[^{35}S]methionine labeling of mitochondrial gene products. Cells were grown in the presence of [^{35}S]methionine and cycloheximide, and the labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (49). Prestained protein molecular weight standards were obtained from Gibco BRL, Gaithersburg, Md.

RESULTS

Construction of *COB* deletion strains. Translation of cytochrome *b* in yeast mitochondria involves interactions between the 5′ UTR of *COB* mRNA, the mRNA-specific translational activators, and the ribosomes. To determine which regions of the 954-nucleotide 5′ UTR are important for these interactions, we constructed yeast strains with deletions in *COB* (Fig. 1; Materials and Methods), and analyzed the effects of the deletions on translation of *COB* transcripts. This analysis focused on the 5′ UTR sequence 3′ of −700 (relative to the ATG at +1), as a previous study showed that the sequence between the 5′ end of the mRNA and −898 is required for *COB* transcript stability and that the sequence between −898 and −707 is not required for *COB* expression (50). As diagrammed in Fig. 1, most of the *COB* deletion strains contained the long form of the *COB* gene, which is composed of six exons and five introns, bi1 through bi5 (16). Three of the strains (682L/170R, 232L/60R-1, and TG955 898L/282R) contained the short form of the *COB* gene, which lacks introns bi1, bi2, and bi3 (Fig. 1) (56). The presence of the expected *COB* deletion in mitochondrial DNA isolated from each of the deletion strains was verified by Southern blotting (data not shown) and by determination of the sequence surrounding the *Eco*RI site at each of the deletion junctions (summarized in Materials and Methods). In each case, the data indicated that the expected deletion was present in the 5′ UTR of an otherwise wild-type *COB* gene.

The sequence between −232 and −60, and between −33 and −4 is important for *COB* expression. If the *COB* 5′ UTR sequence required for cytochrome *b* synthesis was deleted, the cells should be unable to respire. Therefore, the respiratory capability of each *COB* deletion strain was determined by testing its ability to grow on medium containing glycerol as the sole carbon source (Fig. 2); growth on glycerol requires a functional respiratory chain.

Strains 682L/282R, 682L/170R, and 104L/60R grew like the wild type on glycerol, while strain TG955 898L/282R showed somewhat slower growth. In contrast, strains 682L/4R, 104L/4R, 33L/4R, and 232L/60R-2 did not grow on glycerol, while strain 232L/60R-1 grew extremely slowly. These results indicate that sequences between −232 and −60 and between −33 and −4 are important for *COB* expression.

The mutant phenotypes of the respiration-incompetent deletion strains may have resulted from unexpected mutations that occurred outside the sequenced regions during construction of the strains rather than from the deletions in *COB*. Therefore, the mutations responsible for the phenotypes were mapped by mating the respiratory negative strains to petite strains that carried only small portions of the *COB* gene (data not shown). If the petite mitochondrial genome was sufficient for recombinational repair of the mutation, then the resulting diploids were competent for respiration. For each of the respiration-negative strains, the mutation responsible for the mutant phenotype mapped to the region of the *COB* 5′ UTR that included the introduced deletion.

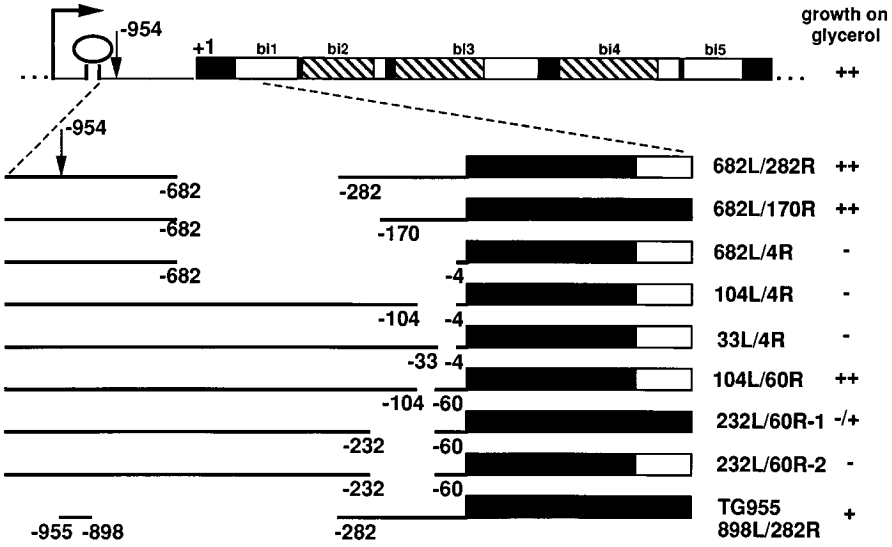

FIG. 1. The wild-type *COB* transcription unit and deletions constructed within the *COB* 5′ UTR. The long form of the wild-type mitochondrial *COB* gene, encoding cytochrome *b*, is diagrammed on the top line. Transcription begins at the *COB* promoter (rightward arrow) and proceeds through $tRNA^{Glu}$ (open circle) and *COB* sequence. The first base pair of the *COB* coding sequence is numbered +1. The long form of the *COB* gene contains six exons (solid bars) and five introns (labeled bi1 through bi5). The short form of the *COB* gene lacks introns bi1, bi2, and bi3. Introns bi2, bi3, and bi4 contain open reading frames (striped bars) that encode maturases required for excision of the encoding intron. Noncoding sequence within the introns is indicated by the open bars. In wild-type strains, the initial *COB* transcript is spliced, cleaved at nucleotide −954 or −955 (downward arrow), and cleaved at the 3′ end to yield the mature 2.2-kb *COB* mRNA. Below the wild-type *COB* transcription unit, the 5′ UTR of *COB* is expanded and the deletions constructed within the 5′ UTR are diagrammed. The deletion endpoints are labeled according to the first wild-type base pair at that endpoint. In each construct, the deleted sequence was replaced with an *Eco*RI site, with the exception of deletion 682L/170R, in which the deleted sequence was replaced with a 24-bp linker sequence that included an *Eco*RI site. Note that strains 682L/170R, 232L/60R-1, and TG955 898L/282R contain the short form of the *COB* gene; exons 1 through 4 are contiguous in these strains. The relative (to wild-type) growth on glycerol of each *COB* deletion strain (see Fig. 2) is indicated on the right. Growth on glycerol requires a functional respiratory chain.

Sequence elements between −232 and −4 are important for translation of *COB* transcripts. The deletions within the *COB* 5′ UTR could lead to respiratory incompetence by affecting the stability, processing, or translation of *COB* transcripts. In strains with a primary defect in the 5′ processing and/or stability of *COB* transcripts, *COB* transcripts are not detectable by Northern blot (17, 50, 66). In contrast, defects in translation result in the accumulation of *COB* transcripts that are only partially spliced. The partially spliced transcripts accumulate because introns bi2, bi3, and bi4 contain open reading frames that encode maturases required for the excision of the encoding intron (2, 3, 15, 29, 32, 35, 39, 41, 73). These maturase-encoding open reading frames are in frame with the cytochrome *b* coding sequence, and the maturases are translated as fusion proteins from the *COB* AUG at +1. Therefore, mutations that eliminate translation of *COB* transcripts also elimi-

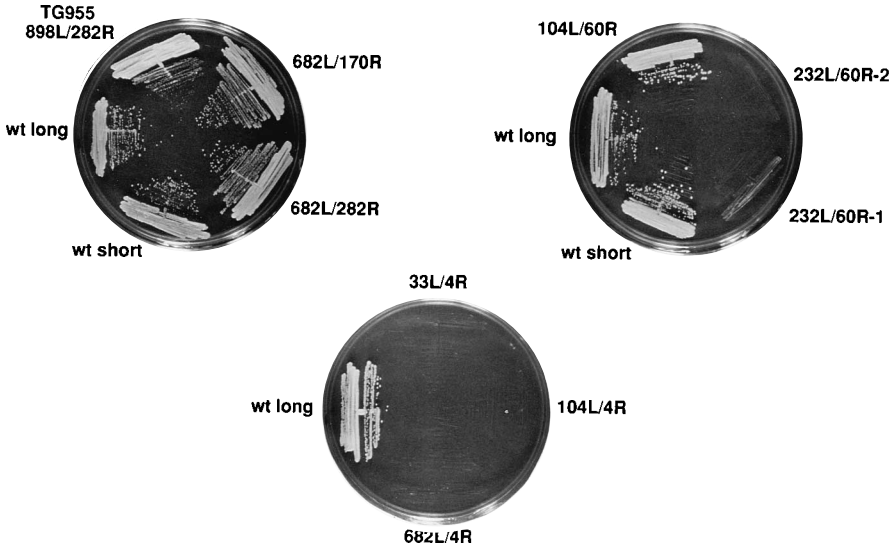

FIG. 2. Respiratory growth of *COB* deletion strains. Wild-type strains A21 (wt short) and LL20/KL14 (wt long), and the *COB* deletion strains were taken from patches grown overnight on glucose (YPD) and streaked on plates that contained glycerol as the sole carbon source (YPG). Growth on glycerol requires a functional respiratory chain. The plates were incubated at 30°C for 4 days.

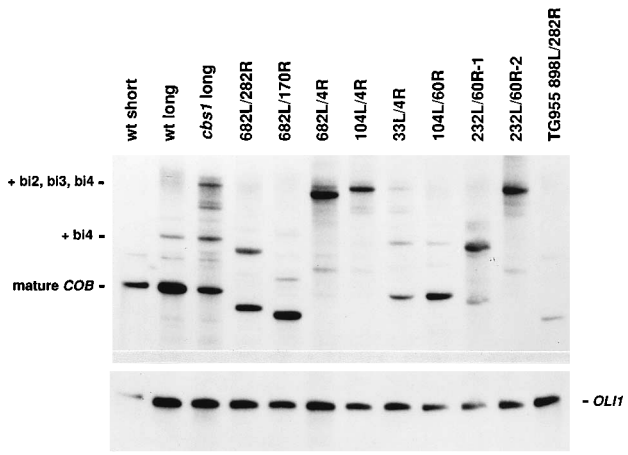

FIG. 3. Northern blot analysis of *COB* transcripts in *COB* deletion strains. Mitochondrial RNA isolated from wild-type strains A21 (wt short) and LL20/KL14 (wt long), from strain N356/KL14 (*cbs1* long), and from the *COB* deletion strains was separated on a nondenaturing agarose gel, transferred to a Nytran membrane, and probed with labeled cob654A oligonucleotide (see Materials and Methods and Table 2). The blot was then stripped of the cob654A probe and reprobed with the *OLI1* sequence (see Materials and Methods). The positions of wild-type *COB* transcripts that are fully spliced (mature *COB*), transcripts that contain intron bi4 only, and transcripts that contain all three maturase-encoding introns (bi2, bi3, and bi4) are indicated on the left. That the indicated transcripts did contain bi4 was verified by hybridization to a bi4-specific probe (see Materials and Methods). Analogous transcripts from the *COB* deletion strains varied in size as a result of the deletions in the 5′ UTR. The signals obtained from the *OLI1* probe are shown below those obtained from the *COB* probe. To determine the relative (to wild-type) steady-state level of *COB* transcripts in each of the strains, the signals obtained from *COB* transcripts were quantitated with a Betascope Analyzer and normalized to the signal obtained from *OLI1* transcripts in the same strain. Data obtained from quantitation of the Northern blot signals are summarized in Table 3.

nate maturase synthesis and excision of the maturase-encoding introns. For example, in *cbs1* and *cbs2* mutant strains, *COB* transcripts are not translated, and the resulting lack of maturase synthesis leads to the accumulation of transcripts containing introns bi2, bi3, and bi4 (52, 58). In addition to its role in bi4 excision, the bi4 maturase is required in *trans* for splicing of the fourth intron (ai4) of mitochondrial *COX1* transcripts, encoding subunit 1 of cytochrome oxidase (15). Therefore, the steady-state level of mature *COX1* mRNA is a second, indirect measure of bi4 maturase activity.

To determine whether the primary defect in the *COB* deletion strains was in translation of *COB* transcripts, the steady-state levels of mature and partially spliced *COB* and *COX1* transcripts were measured. Northern blots of mitochondrial RNA isolated from the deletion strains were analyzed with *COB*-specific (Fig. 3) and *COX1*-specific (data not shown) probes, and the signals obtained from partially spliced and mature *COB* transcripts and from mature *COX1* transcripts were quantitated and compared with those of wild-type strains (Table 3). In the respiration-incompetent strains 682L/4R, 104L/4R, and 232L/60R-2 (long form), no mature *COB* mRNA was detected, but more slowly migrating *COB* transcripts did accumulate. Two observations suggest that these larger transcripts were partially spliced transcripts that contained the maturase-encoding introns bi2, bi3, and bi4: (i) these transcripts migrated similarly to the largest transcript in the *cbs1* mutant strain, which is defective in the excision of the maturase-encoding introns (58), and (ii) these transcripts hybridized to a bi4-specific probe (data not shown; see Materials and Methods). That transcripts retaining the maturase-encoding introns accumulated in strains 682L/4R, 104L/4R, and 232L/60R-2 (long form) suggests that the deletions lead to a defect in translation of *COB* transcripts. Consistent with this hypothesis is the observation that no mature *COX1* transcripts were detected in strain 682L/4R, 104L/4R, or 232L/60R-2.

Mature *COB* and *COX1* mRNAs were not detected in the respiration-incompetent strain 232L/60R-2 (long form); however, in strain 232L/60R-1 (short form), which grew extremely slowly on glycerol, low levels of the mature *COB* and *COX1* mRNAs were present (Table 3). In both strains, transcripts containing the maturase-encoding introns accumulated. The simplest interpretation of these data is that the −232 to −60 deletion impairs translation of *COB* transcripts, resulting in limiting levels of the maturases. Because strain 232L/60R-2 contains three maturase-encoding introns (bi2, bi3, and bi4)

TABLE 3. Steady-state levels of *COB* and *COX1* transcripts

Strain	Glycerol growth[a]	% Mature *COB* transcripts[b]	% Total *COB* transcripts[c]	Partially spliced/ mature ratio[d]	% Mature *COX1* transcripts[e]
wt short[f]	++	100	100	100	100
wt long[f]	++	100	100	100	100
682L/282R	++	40 ± 5	58 ± 5	693 ± 93	53 ± 18
682L/170R	++	63 ± 25	67 ± 26	138 ± 2	113 ± 30
682L/4R	−	—[g]	46 ± 4	—	—
104L/4R	−	—	59 ± 17	—	—
33L/4R	−	27 ± 3	48 ± 6	1,686 ± 115	21 ± 7
104L/60R	++	85 ± 9	86 ± 10	132 ± 12	56 ± 19
232L/60R-1	±	10 ± 5	58 ± 17	4,078 ± 615	12 ± 7
232L/60R-2	−	—	49 ± 2	—	—
TG955 898L/282R	+	6 ± 3	9 ± 4	254 ± 46	40 ± 12

[a] Relative growth of single colonies on glycerol, which requires respiratory growth.
[b] Steady-state level of the fully spliced *COB* transcript, determined by Northern blot analysis (see Fig. 3 and Materials and Methods) and expressed as a percentage of the level of the wild-type *COB* transcript. Percentages were obtained from quantitation of two Northern blots and were averaged; standard deviations are given.
[c] Combined steady-state levels of the fully spliced and partially spliced *COB* transcripts.
[d] Ratio of the steady-state level of partially spliced *COB* transcripts (containing introns bi2, bi3, and bi4; or bi3 and bi4; or bi4 only) to that of the fully spliced *COB* transcript.
[e] Steady-state level of the fully spliced (1.9-kb) *COX1* transcript.
[f] Transcript levels in *COB* deletion strains containing the short form of the *COB* gene (682L/170R, 682L/282R-1, and TG955 898L/282R) were compared with those in strain A21, which contains the short form of the wild-type *COB* gene (wt short). Transcript levels in all other *COB* deletion strains were compared with those in strain LL20/KL14, which contains the long form of the wild-type *COB* gene (wt long).
[g] —, transcripts were not detected.

while strain 232L/60R-1 contains only one (bi4), strain 232L/60R-2 is probably more sensitive to the defect in translation.

In the respiration-competent deletion strains 682L/282R, 682L/170R, and 104L/60R, the steady-state levels of the mature 2.2-kb *COB* mRNA and of the mature *COX1* mRNA were at least 40% those in the wild type, indicating that maturase production and thus translation are relatively normal in these strains. However, in strain 682L/282R, intron excision was slightly retarded, as the ratio of partially spliced to fully spliced transcripts was six- to eightfold greater than that in the wild type, suggesting some involvement of the deleted sequence in translation of *COB* transcripts. In strain TG955 898L/282R, the ratio of partially spliced to mature mRNA was only two- to threefold greater than that in the wild type, suggesting that *COB* transcripts are translated. However, *COB* transcripts (mature plus partially spliced) accumulated to only 9% of the wild-type level, indicating that the large deletion of the *COB* 5′ UTR sequence results in transcript degradation. The low steady-state level of mature *COB* transcripts (approximately 6% that of the wild type) explains the slightly slowed respiratory growth of this strain (50, 66).

The data obtained from the respiration-competent strains indicate that the sequence from −898 to −282, from −682 to −170, and from −104 to −60 is not required for translation of *COB* transcripts when the remainder of the 5′ UTR is present. Combined with the observation that the deletion of sequence from −232 to −60 or from −104 to −4 led to extremely inefficient translation, these data suggest a role for the sequences between −170 and −104 and between −60 and −4 in translation of *COB* transcripts.

In strain 33L/4R, the levels of mature *COB* and *COX1* mRNAs were approximately 27 and 21% those in the wild type, respectively, indicating that the maturases were synthesized. While these data suggest that *COB* mRNA is translated in strain 33L/4R, the cells were unable to respire. A testable hypothesis consistent with these data was that in strain 33L/4R, maturases are produced but cytochrome *b* protein is nonfunctional.

Correct start codon selection requires the sequence between −33 and −4. To verify that *COB* transcripts are not efficiently translated in strains 682L/4R, 104L/4R, 232L/60R-1, and 232L/60R-2 and to determine whether cytochrome *b* is synthesized in strain 33L/4R, mitochondrial gene products from the deletion strains were labeled in vivo with [^{35}S]methionine and analyzed on a denaturing polyacrylamide gel (Fig. 4). As expected, cytochrome *b* that migrated at the same rate as in the wild type was present in the respiration-competent strains 682L/282R, 682L/170R, and 104L/60R and to a lesser extent in strain TG955 898L/282R. In contrast, cytochrome *b* protein was not detected in strains 682L/4R, 104L/4R, 232L/60R-1, and 232L/60R-2, suggesting that *COB* transcripts are not translated. Labeled maturases were not observed in strains that accumulated high steady-state levels of the maturase-encoding transcripts (strains 682L/4R, 104L/4R, 232L/60R-1, and 232L/60R-2). This observation is consistent with the hypothesis that the primary defect in these strains is in translation of *COB* transcripts and not in splicing.

In strain 33L/4R, no wild-type cytochrome *b* was detected, but a novel protein that migrated slightly faster than wild-type cytochrome *b* was observed. These data are consistent with the hypothesis that translation initiated at the next in-frame AUG at position +96, resulting in a protein that lacks 31 N-terminal amino acids and is 3.4 kDa smaller than wild-type cytochrome *b*. The truncated protein must be nonfunctional because strain 33L/4R is unable to respire. Initiation at an in-frame AUG within the first two exons of *COB* would also explain the ability of strain 33L/4R to synthesize the intron-encoded maturases and thus the mature *COB* and *COX1* mRNAs (Fig. 3; Table 3).

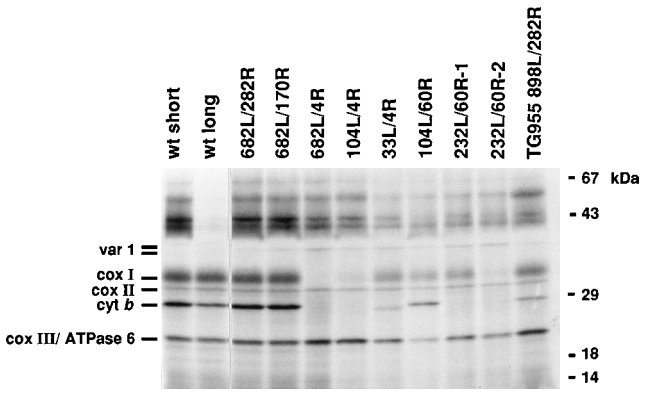

FIG. 4. [^{35}S]methionine-labeled mitochondrial gene products in *COB* deletion strains. Mitochondrial gene products in the wild-type strains A21 (wt short) and LL20/KL14 (wt long) and in the *COB* deletion strains were labeled with [^{35}S]methionine in the presence of cycloheximide, an inhibitor of cytoplasmic translation (see Materials and Methods). Mitochondria isolated from each strain were suspended in Laemmli buffer, and the labeled proteins were separated on an SDS–7.5 to 15% polyacrylamide gradient gel. The positions of major mitochondrial gene products are indicated on the left, while the positions and sizes of protein molecular mass standards (see Materials and Methods) are indicated on the right.

To test the hypothesis that the novel protein detected in strain 33L/4R was translated from *COB* transcripts, mitochondrial gene products in a *cbp1* mutant strain that contained the 33L/4R mitochondrial genome were labeled in vivo (Fig. 5). The product of the nuclear *CBP1* gene is required for *COB* expression; in *cbp1* mutant strains, *COB* transcripts are specifically degraded (17). The novel protein was not detected in the *cbp1*/33L-4R strain (CP1L/33L-4R), consistent with the hypothesis that it is a truncated form of cytochrome *b*. An alternative hypothesis is that the novel protein is translated from *COX1* transcripts, which are indirectly affected by the lack of bi4 maturase in *cbp1* mutant strains. However, two observations argue against this alternative: (i) both mature *COX1* mRNA and wild-type Cox1 protein are present in strain 33L/4R (Fig. 4; Table 3), indicating that *COX1* expression is

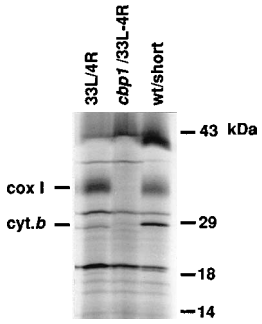

FIG. 5. [^{35}S]methionine-labeled mitochondrial gene products in a *cbp1* mutant strain carrying the 33L/4R mitochondrial genome. Mitochondrial gene products in the wild-type strain A21 (wt/short), strain 33L/4R, and strain CP1L/33L-4R (*cbp1*/33L-4R) were labeled with [^{35}S]methionine in the presence of cycloheximide, an inhibitor of cytoplasmic translation (see Materials and Methods). Mitochondria isolated from each strain were suspended in Laemmli buffer, and the labeled proteins were separated on an SDS–7.5 to 15% polyacrylamide gradient gel. The positions of major mitochondrial gene products are indicated on the left, while the positions and sizes of protein molecular mass standards (see Materials and Methods) are indicated on the right.

relatively normal, and (ii) the novel protein was not detected in strain 232L/60R-1, in which the levels of *COX1* mRNA and protein are similar to those in strain 33L/4R (Fig. 4; Table 3). Together, the data suggest that the sequence between −33 and −4 is not crucial for translation initiation but is important for selection of the correct start codon.

The *COB* deletion strains are dependent on *CBS2* function. It was possible that in the respiration-competent deletion strains, the deletion of *COB* 5′ UTR sequence produced novel mRNAs that do not require the mRNA-specific factors Cbs1p and Cbs2p that are required for translation of wild-type *COB* mRNA (61, 63). To determine whether translation remained dependent on Cbs2p in strains containing the 682L/170R, 104L/60R, and TG955 898L/282R mitochondrial genomes, the deletion strains were mated to the *cbs2* mutant strains W303Δcbp7(cbs2)/*rho*^{0} and E67/*rho*^{0}, the diploids were sporulated and colonies from dissected tetrads were tested for their ability to grow on glycerol (data not shown). If the mutant mitochondrial genomes suppress the *cbs2* (*cbp7*) mutations, all four spores from each tetrad should be respiration competent. If, however, the mutant mitochondrial genomes behave like the wild-type genome, respiratory competence should segregate 2:2 in each tetrad. In 100% of the tetrads obtained from each cross, respiratory competence segregated 2:2 (the average number of tetrads analyzed was 18 for crosses to W303Δ*cbp7*/*rho*^{0} and 6 for crosses to E67/*rho*^{0}). All of the spores were able to form respiration-competent diploids when mated to a *rho*^{0} strain, indicating that mitochondrial genomes had segregated to each spore. As expression of *COB* from the mutant mitochondrial genomes remained dependent on Cbs2p, it is likely that the requirements for translation of the mutant *COB* transcripts are similar to those of wild-type transcripts.

A putative Shine-Dalgarno-like sequence is not required for *COB* expression. In an analysis of the mitochondrial gene encoding the 15S rRNA, Li et al. (42) identified regions of complementarity between the 3′ end of the 15S rRNA and the 5′ UTRs of several mitochondrial mRNAs. The complementarity in *COB* transcripts extends from nucleotides −107 through −104. As the analysis of the deletion strains indicated that the sequence between −232 and −60 is important for translation of *COB* transcripts, strains MSD2-1 and MSD2-4 were constructed (see Materials and Methods) and tested for their ability to respire (Fig. 6). Strains MSD2-1 and MSD2-4, derived from independent mitochondrial transformants, contained *COB* sequence identical to that in the respiration-competent strain 104L/60R, except that positions −107 through −104 had been changed from TAAG to ATTC. Single colonies of strains MSD2-1 and MSD2-4 both showed respiratory growth similar to that of the wild type and strain 104L/60R. This result indicates that the putative Shine-Dalgarno-like sequence is not crucial for the translation of *COB* transcripts. Other sequence elements within the −232 to −60 interval may be important for interactions between the translational machinery and *COB* mRNA.

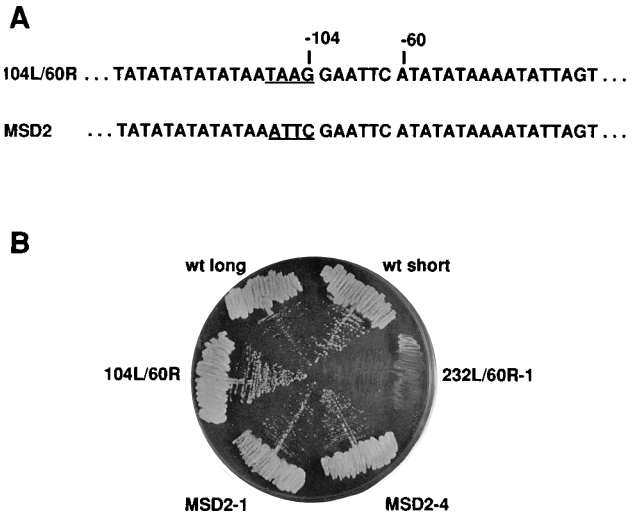

FIG. 6. Phenotype of strains MSD2-1 and MSD2-4. (A) The sequence surrounding the deletion junction in the respiration-competent strain 104L/60R is shown on the top line, and the deletion endpoints (at positions −104 and −60) are indicated; note that the deleted sequence was replaced with an *Eco*RI site (GAATTC). In the MSD2 strains (MSD2-1 and MSD2-4), the *COB* 5′ UTR sequence was identical to that of strain 104L/60R, except that nucleotides complementary to the 3′ end of the 15S rRNA (underlined) were changed from TAAG to ATTC. (B) Wild-type strains A21 (wt short) and LL20/KL14 (wt long) and the *COB* deletion strains were taken from patches grown overnight on glucose (YPD) and streaked on plates that contained glycerol as the sole carbon source (YPG). Growth on glycerol requires a functional respiratory chain. The plates were incubated at 30°C for 4 days.

DISCUSSION

Mitochondrial mRNAs in *S. cerevisiae* have long AU-rich 5′ UTRs that interact with mRNA-specific translational activators and mitochondrial ribosomes. In an initial step toward understanding the requirements for these protein-RNA interactions, we have analyzed the effects of deletions in the 5′ UTR of the mitochondrial *COB* gene, encoding cytochrome *b*, on translation of *COB* transcripts in vivo. The deletion of *COB* 5′ UTR sequences between positions −232 and −60 (relative to the AUG at +1) or between positions −104 and −4 led to a severe reduction in the levels of cytochrome *b* following in vivo labeling of mitochondrial gene products. Reduced production of intron-encoded maturases in strains carrying either the −232 to −60 or the −104 to −4 deletion, as evidenced by low levels of mature *COB* and *COX1* mRNAs, indicated that the primary defect was in translation of *COB* transcripts.

The −232 to −60 and −104 to −4 deletions could affect *COB* translation by removing a binding site for ribosomes and/or the *COB*-specific translational activators, Cbs1p and Cbs2p. Alternatively, the deletions might alter the three-dimensional structure of the RNA such that a crucial binding site is no longer recognized by the translational machinery. In either case, the affected binding site must lie between −170 and −104 or between −60 and −33 (unless the binding site is redundant), because deletion of the surrounding portions of the *COB* 5′ UTR did not seriously affect translation. At present, we cannot determine whether the identified regions of the 5′ UTR play structural roles or directly bind components of the translational apparatus. In vivo analysis of the structure of *COB* transcripts or in vitro binding studies may distinguish between these possibilities.

The results of previous studies on translation of mitochondrial mRNAs other than *COB* have also implicated sequences within the 5′ UTR that are not immediately adjacent to the AUG. The insertion of a T residue at position −88 of *OLI1* mRNA, encoding ATP synthase subunit 9, led to a loss of subunit 9 synthesis at high temperature (57). Translation of a hybrid *COX3-COB* mRNA, in which *COX3* sequence 5′ of −173 was fused to *COB* sequence downstream of −5, was dependent on the *COX3*-specific translational activators Pet54p, Pet494p, and Pet122p (7). Also, translation of a mutant *COX3* gene that retained only a portion of the 5′ UTR (from the 5′ end at −612 to −437 and from −359 to −329) remained dependent on the *COX3*-specific translational acti-

vators (9). These data indicate that Pet54p, Pet494p, and Pet122p act via the 5′ half of the *COX3* 5′ UTR, upstream of −329.

Within the −170 to −104 and the −60 to −33 intervals defined by the deletions, there are no immediately apparent sequence features suggestive of a potential binding site for either the mitochondrial ribosomes or Cbs1p and Cbs2p. Biochemical and genetic evidence indicates that mRNA-specific translational activators in *Chlamydomonas reinhardtii* bind to stem-loop structures within the 5′ UTRs of chloroplast mRNAs (13, 59, 60). While several potential stem-loop structures exist between position −104 and the AUG at +1 of *COB* mRNA (identified with the Stemloop program in the Wisconsin Genetics Computer Group package), they would be disrupted by deletions which do not eliminate translation (e.g., in strains 104L/60R and 33L/4R). Potential stem-loop structures also exist between −146 and −107, but a description of any role they may play in translation of *COB* transcripts awaits further analysis. Nucleotides −107 through −104 have the potential to base-pair with 4 of the 10 nucleotides in the mitochondrial 15S rRNA that are complementary to sequences within the 5′ leaders of several mitochondrial mRNAs (42). However, mutation of these bases did not lead to a readily observable effect on the respiratory capability of the cells, indicating that they are not crucial for translation of *COB* transcripts. In contrast, deletion of the Shine-Dalgarno sequence of prokaryotic mRNAs leads to a drastic reduction in translation (25, 34, 65). Shine-Dalgarno-like complementarity between *COB* transcripts and the 15S rRNA may involve other sequences within the defined regions of the 5′ UTR or may not be as important for translation of mitochondrial mRNAs as for that of prokaryotic mRNAs.

Perhaps the most intriguing phenotype was that of strain 33L/4R. In this strain, the deletion of the sequence between −33 and −4 did not abolish translation of *COB* transcripts but led to the synthesis of a novel protein that is a truncated form of cytochrome *b*. Both the apparent size of the novel protein and the presence of *COB*-encoded maturases in strain 33L/4R are consistent with the interpretation that translation of the 33L/4R *COB* transcripts is initiated at the first in-frame AUG (at +96) downstream of the wild-type initiation codon. The possibility that protein synthesis is also initiated at the out-of-frame AUGs at positions +20 and +87 cannot be excluded, because the resulting peptides would be too small to have been detected in the in vivo labeling experiment. Initiation at out-of-frame AUGs could explain the lower levels of *COB* and *COX1* expression observed in strain 33L/4R. Regardless, the data suggest a role for the sequence between −33 and −4 in selection of the correct AUG.

How might the sequence just 5′ of the start codon direct mitochondrial ribosomes to begin protein synthesis at the correct AUG? One possibility is that this sequence provides the correct spacing between an upstream binding site for the mRNA-specific translational activators or the mitochondrial ribosomes and the AUG at +1. Once positioned, the ribosomes either would begin protein synthesis at a specified distance downstream or might scan downstream for the first appropriate start site. Alternatively, the sequence between −33 and −4 might be part of a binding site for ribosomes or for Cbs1p or Cbs2p. However, if recognition by mitochondrial ribosomes or Cbs1p or Cbs2p requires a specific sequence or structure just 5′ of the AUG at +1, a similar sequence or structure must exist just 5′ of the AUG at +96 in strain 33L/4R. When the sequences surrounding these two AUGs are aligned, there are no obvious similarities. In light of this observation, we wonder whether translation from the +96 AUG is dependent on Cbs1p and Cbs2p. Understanding the role of the sequence just 5′ of the AUG in translation awaits further mutational analyses or the development an in vitro system for mitochondrial translation.

Taken together, the data are consistent with a hypothesis in which mitochondrial ribosomes and the mRNA-specific translational activators, Cbs1p and Cbs2p, bind to sites on *COB* transcripts either between −170 and −104 or between −60 and the AUG at +1. The data do not exclude the possibility that sequence important for *COB* transcript stability, extending from the 5′ end of the mRNA, at −954, to position −898, also interacts with the translational machinery. In addition to the certain involvement of Cbs1p, Cbs2p, and the general translation machinery in expression of *COB* mRNA, isocitrate dehydrogenase binds to the 5′ UTR sequence between −343 and −26 and to other mitochondrial mRNA leaders (14). The association of isocitrate dehydrogenase with the mRNAs has been proposed to be regulatory rather than required (20), and since the interaction site overlaps with that defined by this study, it will be interesting to see how this additional factor affects translation of *COB* mRNA.

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