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Site-Directed Mutagenesis of a *Saccharomyces cerevisiae* Mitochondrial Translation Initiation Codon

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ABSTRACT

We have used a generally applicable strategy for gene replacement in yeast mitochondria to mutate the translation initiation codon of the *COX3* gene from AUG to AUA. The mutation, *cox3-1*, substantially reduced, but did not eliminate, translation of cytochrome *c* oxidase subunit III (coxIII). Strains bearing the mutation exhibited a leaky (partial) nonrespiratory growth phenotype and a reduced incorporation of radiolabeled amino acids into coxIII *in vivo* in the presence of cycloheximide. Hybridization experiments demonstrated that the mutation had little or no effect on levels of the *COX3* mRNA. Residual translation of the *cox3-1* mutant mRNA was dependent upon the three nuclearly coded mRNA-specific activators *PET494*, *PET54* and *PET122*, known from previous studies to work through a site (or sites) upstream of the initiation codon to promote translation of the wild-type mRNA. Furthermore, respiratory growth of *cox3-1* mutant strains was sensitive to decreased dosage of genes *PET494* and *PET122* in heterozygous mutant diploids, unlike the growth of strains carrying wild-type mtDNA. Some residual translation of the *cox3-1* mRNA appeared to initiate at the mutant AUA codon, despite the fact that the 610-base 5'-mRNA leader contains numerous AUA triplets. We conclude that, while AUG is an important component of the *COX3* translation initiation site, the site probably is also specified by other sequence or structural features.

MITOCHONDRIAL translation involves a collaboration between mitochondrial and nuclear-encoded gene products. In *Saccharomyces cerevisiae*, the mitochondrial genome is known to contribute the large and small rRNAs, a full set of tRNAs, an RNA required for tRNA processing, and one mitochondrial ribosomal protein to the mitochondrial translation machinery (reviewed in ATTARDI and SCHATZ 1988; COSTANZO and FOX 1990; DUJON 1981; GRIVELL 1989; TZAGOLOFF and DIECKMANN 1990). All other proteins known to participate in mitochondrial translation are encoded in the nucleus, synthesized in the cytoplasm and imported into the organelle (ATTARDI and SCHATZ 1988; COSTANZO and FOX 1990; DUJON 1981; GRIVELL 1989; TZAGOLOFF and DIECKMANN 1990).

Translation initiation appears to be a particularly important point of control in the expression of yeast mitochondrial genes, since translation of at least five of the eight major mitochondrial mRNAs requires, or appears to require, nuclearly coded mRNA-specific activators (ACKERMAN *et al.* 1991; COSTANZO and FOX 1986, 1988; COSTANZO, SEAVER and FOX 1986; DECOSTER *et al.* 1990; DIECKMANN and TZAGOLOFF 1985; FOX *et al.* 1988; KLOECKENER-GRUISSEM, MCEWEN and POYTON 1988; MÜLLER *et al.* 1984; POUTRE and FOX 1987; RÖDEL 1986; RÖDEL and FOX 1987). In the best understood case, that of the cytochrome *c* oxidase subunit III (coxIII) mRNA, translation depends on three activators coded by nuclear genes (COSTANZO and FOX 1986; COSTANZO, SEAVER and FOX 1986; FOX *et al.* 1988; KLOECKENER-GRUISSEM, MCEWEN and POYTON 1988; MÜLLER *et al.* 1984) that work through a site (or sites) in the upstream portion of the 5'-mRNA-leader (COSTANZO and FOX 1988). At least one of the mRNA-specific factors interacts functionally with the small subunit of mitochondrial ribosomes (HAFFTER, MCMULLIN and FOX 1990, 1991; MCMULLIN, HAFFTER and FOX 1990). However, the mechanism of positive control of translation in this system remains to be determined.

Even more poorly understood are the general features of translation initiation in yeast mitochondria, which do not appear to resemble closely those of either prokaryotic or eukaryotic cytoplasmic systems (KOZAK 1983). There does not seem to be a Shine-Dalgarno-like interaction (reviewed by GOLD 1988) between the small subunit rRNA and the mRNA, since sequences complementary to the 3'-domain of the small rRNA cannot be found in the 5'-mRNA-leaders uniformly close to the initiation codon (COSTANZO and FOX 1990; LI *et al.* 1982). Furthermore, it appears unlikely that yeast mitochondrial ribosomes identify initiation codons by "scanning" for AUG from the 5'-end (CIGAN, FENG and DONAHUE 1988; KOZAK 1989), since seven of the eight major mitochondrial mRNAs have long 5'-leaders (ranging from roughly 300 to 950 bases; see GRIVELL 1989) that each contain at least

one open reading frame upstream of the protein coding sequence. Indeed, the only obvious general signal for translation initiation in all eight major mitochondrial mRNAs is the use of AUG as an initiation codon.

We have begun a genetic examination of the general features of yeast mitochondrial translation by mutating the initiation codon of the mitochondrial gene that encodes coxIII. (We refer here to wild-type and mutant alleles of this gene as *COX3* and *cox3*, respectively; see COSTANZO and FOX 1990). This gene codes for an mRNA with a "typical" 5′-leader that is rich in A (49%) and U (46%), and is approximately 610 bases long (KLOECKENER-GRUISSEM, MCEWEN and POYTON 1988; MÜLLER *et al.* 1984; THALENFELD and TZAGOLOFF 1980). Although the coxIII polypeptide has not been directly sequenced, the initiation codon has been identified by comparing the predicted N-terminal coxIII sequences of human, bovine and *Neurospora crassa* to that of *S. cerevisiae* (BROWNING and RAJBHANDARY 1982; THALENFELD and TZAGOLOFF 1980).

We describe here a mutation that changes the *COX3* initiation codon to AUA. While AUA encodes Met in yeast mitochondria (HUDSPETH *et al.* 1982), it is known to be a poor initiation codon in other systems (DONAHUE and CIGAN 1988; SHERMAN and STEWART 1982; SHINEDLING *et al.* 1987). Furthermore, AUA would appear to be inconspicuous at the end of the A + U-rich *COX3* mRNA-leader, which contains 98 AUA triplets.

We have used a generally applicable two-step mitochondrial transformation/gene replacement strategy to incorporate the *in vitro*-generated mutant allele into otherwise wild-type mtDNA. Interestingly, the AUA initiation codon mutation significantly reduced, but did not abolish, the translation of the message. Our data indicate that translation initiates inefficiently at the mutant AUA codon, suggesting that ribosomal recognition of the initiation site in the wild-type *COX3* mRNA may involve sequence or structural features in addition to the initiation codon.

MATERIALS AND METHODS

Yeast strains, media and genetic methods: *S. cerevisiae* strains used in this study are listed in Table 1. The mitochondrial DNA in all strains was derived from D273–10B (ATCC 25627; MÜLLER *et al.* 1984). The nuclear genomes of TF189rho° and the mitochondrial transformants LSF2, MCC237 and MCC600 are isogenic to strain DBY947 (NEFF *et al.* 1983). All other strains are nuclearly isogenic or congenic to strain D273–10B, except the diploids LSF18 and LSF20, which are hybrids of these two nuclear backgrounds generated by mating LSF2 with DL1.

Complete medium (1% yeast extract, 2% peptone) with 2% glucose (YPD), with 2% galactose (YPGal) or with 3% each ethanol and glycerol (YPEG), minimal medium with glucose (SD) and appropriate supplements were as described (SHERMAN, FINK and HICKS 1986).

Standard genetic procedures were as described (SHERMAN, FINK and HICKS 1986). *rho°* strains were generated by treatment with ethidium bromide (GOLDRING *et al.* 1970). Transfer of mtDNA by cytoduction was accomplished by mating mtDNA donor strains to *rho°* strains, where either strain carried the *kar1-1* mutation (CONDE and FINK 1976). Cells were mixed in liquid and mated for 4 hr at 30° on YPD plates, after which zygotes were isolated by micromanipulation on SD medium supplemented for the mtDNA recipient. Haploid cytoductants were isolated by streaking of zygotic clones.

Nucleic acid manipulation and analysis: Standard DNA manipulations, plasmid DNA isolation, bacterial transformation of *Escherichia coli DH5α* (Bethesda Research Laboratories), agarose gel electrophoresis, blotting, hybridization, and radioactive labeling of probe DNA by nick-translation were as described (MANIATIS, FRITSCH and SAMBROOK 1982). Sequenase (United States Biochemical Corp.) was used for DNA sequence analysis (SANGER, NICKLEN and COULSON 1977). mtDNA was sequenced directly from total yeast DNA as described (FOX *et al.* 1990).

Total yeast DNA was prepared as described (SHERMAN, FINK and HICKS 1986). Total yeast RNA was prepared according to SCHMITT, BROWN and TRUMPOWER (1990) from cells grown in YPGal. RNA-gel-blots were hybridized to ^{32}P-labeled probes: a plasmid identical to pLSF601 but for a single base substitution, and pSPACT, a bacterial plasmid that carries the yeast *ACT1* gene (MARYKWAS and FOX 1989). The results of RNA gel-blot hybridizations were analyzed quantitatively using a Betascope 603 Blot Analyzer (Betagen Corp.).

Site-directed mutagenesis of the *COX3* gene: A 1.9-kilobase *Xba*I fragment that carries the wild-type *COX3* gene of strain D273-10B was obtained from plasmid pMC201 (M. C. COSTANZO, unpublished results) and was cloned into the *Xba*I site of Bluescript KS− (Stratagene) to create pLSF600. The *cox3-1* (AUG to AUA) mutant plasmid pLSF601 (Figure 1) was derived from pLSF600 by *in vitro* site-directed mutagenesis using the Muta-gene kit (Bio-Rad) and an oligodeoxyribonucleotide of sequence 5′-TCTAAATGTGT-TATAA to direct the change.

Sequence analysis of pLSF600 and pLSF601 confirmed the AUG to AUA change, and revealed that both the wild-type *COX3* and *cox3-1* leader sequences (M. C. COSTANZO and L. S. FOLLEY, unpublished results) differed from the published sequence (THALENFELD and TZAGOLOFF 1980) at five positions, as follows: (1) insertion of an A between A_{-521} and G_{-520} of the published sequence; (2) deletion of T_{-403} between C_{-404} and T_{-402}; (3) insertion of T between G_{-290} and C_{-289}; (4) deletion of A_{-85} between G_{-86} and T_{-84}; (5) insertion of T between T_{-53} and A_{-52} (makes 8 T's in a row).

Mitochondrial transformation: Mitochondrial transformation by high velocity microprojectile bombardment (FOX, SANFORD and MCMULLIN 1988; JOHNSTON *et al.* 1988) was performed as described (FOX *et al.* 1990). Ten Petri plates spread with cells of strain TF189rho° were bombarded with an equimolar mixture of the *cox3-1*-carrying plasmid pLSF601 and the *URA3*, 2-μm vector pCGE137 (MÜLLER and FOX 1984). Two of the roughly 900 Ura$^+$ transformants obtained yielded respiring diploids after mating to a *rho$^+$*, *cox3* tester strain, M7583, whose *cox3* mutation maps to the distal half of the gene (C. SENGSTAG and T. D. FOX, unpublished). Ura$^-$ mitotic segregants were isolated from each of the two mitochondrial transformants: one of these is strain LSF2. (The other mitochondrial transformant behaved identically to LSF2 with respect to its mitochondrial genome, but appeared to have suffered a chromosomal inversion or have become a *MAT***a**/*MAT***a** diploid, perhaps as a

TABLE 1

Strains used in this study

Strain	Genotype	Source
TF175	*MAT***a** *ura3-52 leu2-3 leu2-112 his4-519 pet122-5* [*rho*$^+$]	This study
TF189rho^0	*MAT***a** *ade2-101 ura3-52* [*rho*^{0}]	THORSNESS and FOX (1990)
LSF2	*MAT***a** *ade2-101 ura3-52* [*rho*$^-$ *cox3-1*]	This study
LSF18	*MAT***a** ade2-101 ura3-52 LYS2 / *MATα* ADE2 URA3 lys2 [*rho*$^+$]	This study
LSF20	*MAT***a** ade2-101 ura3-52 LYS2 / *MATα* ADE2 URA3 lys2 [*rho*$^+$ *cox3-1*]	This study
LSF72	*MAT***a** *ade2 ura3* [*rho*$^+$ *cox3-1*]	This study
LSF75	*MATα ura3 lys2* [*rho*$^+$ *cox3-1*]	This study
DAU2	*MAT***a** *ade2 ura3* [*rho*$^+$]	COSTANZO and FOX (1988)
DUL2	*MATα ura3 lys2* [*rho*$^+$]	This study
DL1	*MATα lys2* [*rho*$^+$]	This study
DL2	*MAT***a** *lys2* [*rho*$^+$]	M. C. COSTANZO
MCC118	*MAT***a** *lys2* [*rho*$^+$ *cox3-Δ5*]	M. C. COSTANZO
MCC101	*MAT***a** *ade2 pet494-41* [*rho*$^+$]	M. C. COSTANZO
LSF97rho^0	*MATα lys2 pet54-5* [*rho*^{0}]	This study
TWM10-41rho^0	*MAT***a** *ade2 ura3 pet122-6* [*rho*^{0}]	T. W. MCMULLIN
MCC237	*MAT***a** *ade2-101 ura3-52* [*rho*$^-$ *cox3-Δ5*]	M. C. COSTANZO
MCC600	*MAT***a** *ade2-101 ura3-52* [*rho*$^-$ *COX3*]	M. C. COSTANZO

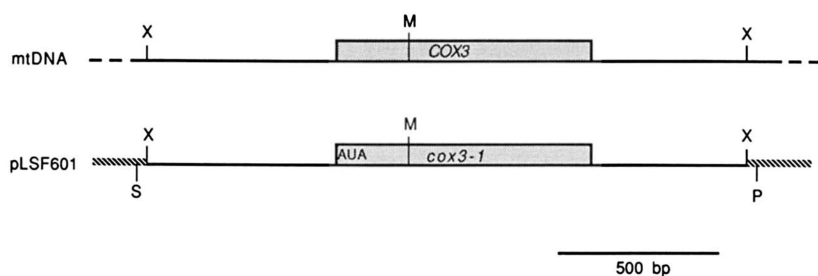

FIGURE 1.—Restriction maps of the *COX3* region in mtDNA and in the *cox3-1* mutant plasmid, pLSF601. *COX3* and *cox3-1* coding sequences are indicated by stippled boxes, flanking mitochondrial sequences by solid lines, and bacterial vector sequences by hatched lines. X, *Xba*I sites endogenous to wild-type mtDNA flanking the *COX3* gene; M, *Mbo*I site; P and S, *Pst*I and *Sac*I sites in vector sequences, each ~25 base pairs from the mtDNA/vector DNA junction in pLSF601.

result of bombardment. It mated well with a *MATα* haploid, yielding one of the strains used in the experiment of Figure 5, but sporulation of this strain yielded very few viable progeny.) MCC600 and MCC237 were obtained by similar mitochondrial transformations of TF189rho^0 with pLSF600 and a plasmid carrying the *cox3-Δ5* mutation (Fig. 4), respectively (M. C. COSTANZO, unpublished results). The *cox3-Δ5* mutation was integrated into wild-type *rho*$^+$ mtDNA via cytoduction to create strain MCC118 (M. C. COSTANZO, unpublished results).

***In vivo* labeling of mitochondrial translation products:** Mitochondrial translation products were radioactively labeled in the presence of cycloheximide as previously described (DOUGLAS and BUTOW 1976; FOX *et al.* 1990; MÜLLER *et al.* 1984). Cells were grown in YP1%D (1% glucose instead of the 2% of standard YPD), and were labeled with the [^{35}S]-*E. coli* hydrolysate labeling reagent "Trans ^{35}S-Label" (ICN Radiochemicals). Labeled proteins from crude mitochondria were electrophoresed through a 9% to 15% (w/v) linear polyacrylamide gradient gel in the presence of sodium dodecyl sulfate (SDS) and autoradiographed.

RESULTS

Replacement of the wild-type *COX3* initiation codon by AUA to create the *cox3-1* mitochondrial mutation: Oligonucleotide mutagenesis was used to convert the *COX3* initiation codon AUG to AUA to generate the allele termed *cox3-1* on plasmid pLSF601 (MATERIALS AND METHODS; Figure 1). The accuracy of the mutagenesis was checked by sequence analysis of the 5′-leader coding region and first third of the structural gene (from the upstream *Xba*I site through 5 codons beyond the *Mbo*I site in structural gene; Figure 1) in pLSF601. Taken together with genetic data presented below, this sequence analysis confirmed that the AUG-to-AUA substitution was the only mutation present in the mtDNA sequences of the plasmid.

To transfer the *cox3-1* allele to a *rho*$^+$ mitochondrial chromosome, we employed a two-step strategy. First, the mutant plasmid was introduced into the mitochondria of a *rho*^{0} recipient. Then, the resulting mitochondrial transformants (synthetic *rho*$^-$ strains) were mated to wild-type (*rho*$^+$), allowing the mutant allele to be transferred to the wild-type mitochondrial genome by homologous double recombination.

In the first step, a strain containing no mtDNA (TF189rho^0) was transformed by high velocity microprojectile bombardment with the mutant plasmid (pLSF601, Figure 1; MATERIALS AND METHODS). Two independent mitochondrial transformants were identified by their ability to form respiring colonies when

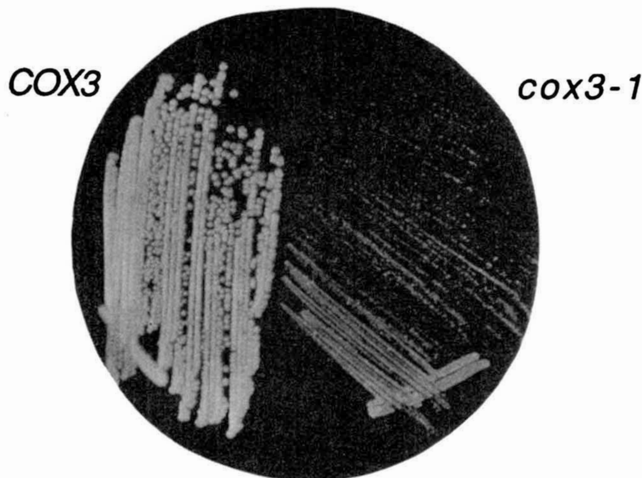

FIGURE 2.—The *cox3-1* mutation causes a leaky nonrespiratory phenotype. Isogenic wild-type *COX3* (LSF18) and *cox3-1* mutant (LSF20) strains were streaked onto medium containing nonfermentable carbon sources (YPEG), and incubated at 30° for 5 days.

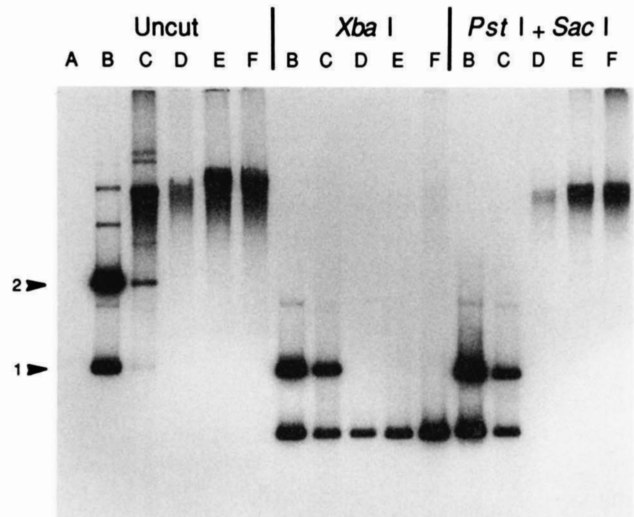

FIGURE 3.—DNA-gel-blot hybridization analysis of a synthetic *rho⁻* transformant and *rho⁺* mutant strains. Total yeast DNA (or plasmid DNA of pLSF601) was isolated from the strains described below, digested as indicated above the lanes, and separated on a 0.8% agarose-Tris-acetate gel. The DNAs were blotted to nitrocellulose, probed with pLSF601 (*cox3-1* plasmid) DNA radioactively labeled by nick-translation, and autoradiographed. Lane A, TF189rho°, the recipient strain prior to transformation; lanes B, pLSF601, the *cox3-1* plasmid used for transformation; lanes C, LSF2, a synthetic *rho⁻* transformant; lanes D, LSF20, a *rho⁺* diploid carrying the integrated *cox3-1* mutation; lanes E, a *rho⁺* haploid carrying the integrated *cox3-1* mutation; lanes F, DUL1, a *rho⁺* wild-type. Arrows 1 and 2 indicate positions of supercoiled plasmid monomer and dimer, respectively, in the "Uncut" lanes. The restriction digests cut pLSF601 (and the DNA of the synthetic *rho⁻* derived from it) into two fragments. The strong upper band in the digested lanes B and C that migrates near the position of arrow 1 is the linear bacterial vector fragment. The lower band common to all the *Xba*I-cut lanes and the similarly migrating band in the *Pst*I + *Sac*I-cut lanes B and C are the *COX3* (or *cox3-1*) fragments diagrammed in Figure 1.

mated to a *cox3* mutant tester strain (marker rescue via mtDNA recombination). The *cox3-1* mtDNA was stably maintained in these mitochondrial transformants; greater than 90% of single-colony subclones derived from each transformant rescued the tester. (As described below, one of these mitochondrial transformants, LSF2, was analyzed fully.)

To replace the *COX3* gene of the wild-type mitochondrial genome with the AUA mutant allele, both mitochondrial transformants were individually mated to the *rho⁺* wild-type strain DL1. Diploids were selected and grown on minimal medium (SD) allowing segregation of homoplasmic parental and recombinant mtDNAs (DUJON 1981). They were then tested for respiration-dependent growth on YPEG medium. While most of the selected diploids had a wild-type respiratory phenotype, about 20% from each cross displayed a leaky (partial) respiration-deficient phenotype. As shown below, this mutant phenotype (Figure 2), which was mitotically stable, resulted from replacement of the *COX3* initiation codon by AUA via homologous recombination and mitotic segregation. The fact that two independent mitochondrial transformants behaved identically when mated to wild type indicates that no spurious mitochondrial mutations were generated during the transformation and integration steps.

Structural analysis of mtDNA in *rho⁻* and *rho⁺* mutant strains: Gel blot analysis (using nick-translated pLSF601 as a probe) demonstrated that most of the plasmid DNA in the mitochondrial transformant LSF2 was concatemerized, but that low levels of supercoiled plasmid monomer and dimer were present (Figure 3). Thus, the mtDNA of LSF2 was similar in structure to previously studied synthetic *rho⁻* strains (FOX, SANFORD and MCMULLIN 1988) and many natural *rho⁻* strains (DUJON 1981). Furthermore, restriction of DNA from strain LSF2 produced fragments identical to those obtained from the plasmid pLSF601 (Figure 3). Thus, the concatemers were arranged head-to-tail, and no gross rearrangements occurred during transformation.

The mtDNA of a *cox3-1* mutant *rho⁺* strain created by gene replacement (diploid strain LSF20, derived by mating LSF2 with DL1) was identical in structure to wild-type *rho⁺* mtDNA by three criteria. First, uncut mtDNA from both LSF20 and wild type migrated as a high molecular weight smear during electrophoresis (Figure 3), with no evidence in the mutant lane of mtDNA monomers or dimers that were present in the synthetic *rho⁻* strain. Second, *Xba*I digestion excised identically sized *COX3*/*cox3-1* fragments from the mtDNAs of the two strains (Figure 3). No hybridization to bacterial vector or other junction fragments was seen, as expected. Finally, the mtDNA of the *rho⁺*, *cox3-1* mutant strain LSF20 did not retain closely flanking restriction sites of the bacterial vector (*Pst*I and *Sac*I, Figures 1 and 3), indicating that the wild-

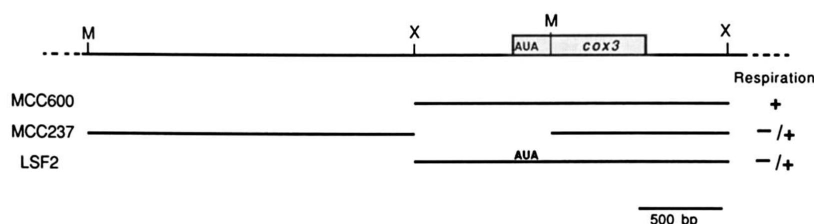

FIGURE 4.—The mutant phenotype maps to the AUA mutation. Haploid *cox3-1* mutant strains (haploids derived by sporulation of the diploid LSF20, and strain LSF75) were mated to the indicated defined *rho*$^-$ strains. Growth of the resulting diploids on medium containing nonfermentable carbon sources (YPEG) was scored as "Respiration:" + = wild type; −/+ = the leaky *cox3-1* phenotype. The top line is a map of the *cox3-1* region in the *rho*$^+$ mutant haploids. Restriction sites are indicated as in Figure 1. The lines below indicate mtDNA sequences carried by the indicated *rho*$^-$ strains. AUA denotes the presence of the *cox3-1* mutation.

type *COX3* initiation codon was replaced via homologous recombination between yeast sequences in the wild-type *rho*$^+$ and *cox3-1 rho*$^-$ mtDNAs. Sequence analysis of the *cox3-1* initiation codon region in the mutant strain confirmed the presence of the AUA mutation (not shown).

The mutant phenotype maps to the AUA codon: Haploid strains bearing the *cox3-1* mutation (see below) were mated to a number of synthetic *rho*$^-$ tester strains that contained defined portions of the *COX3* gene. A *rho*$^-$ tester strain (MCC600) that carried the wild-type initiation region restored wild-type respiration competence to the mutant after mating and mtDNA recombination (Figure 4). Tester *rho*$^-$ strains that either lacked this region (MCC237), or carried the *cox3-1* allele (LSF2) did not rescue the respiration-deficient phenotype of the mutant after mating (Figure 4). Matings to naturally occurring *rho*$^-$ strains carrying portions of the *COX3* gene gave results consistent with these (data not shown). Taken together with the sequence data and transformation results described above, these data confirm that the respiration-defective phenotype results from the AUA substitution mutation.

Substitution of AUA for the *COX3* initiation codon reduces but does not eliminate translation: The substitution of AUA for the wild-type *COX3* AUG initiation codon produced a leaky respiration-deficient phenotype (Figure 2). To test whether the reduced respiration could be ascribed to a reduction in the expression of coxIII protein, mitochondrial translation products were radioactively labeled *in vivo* in the presence of cycloheximide, separated by SDS-gel electrophoresis and visualized by autoradiography (MATERIALS AND METHODS). The *cox3-1* mutant strain LSF20 was compared to an isogenic *COX3* diploid (LSF18), as well as to another *cox3-1* mutant strain derived from the other initial mitochondrial transformant. The expression of coxIII was substantially reduced relative to wild type, but was not abolished by substitution of AUA at the initiation codon (Figure 5). The spectrum of mitochondrial translation products seen in the *cox3-1* mitochondrial mutants was quite similar to that seen in a *COX3* strain that has reduced coxIII expression due to a leaky *pet122* nuclear mutation (Figure 5). Specifically, residual protein at the coxIII position in the nuclear mutant precisely co-migrated with that of the mitochondrial *cox3-1* mutant. Thus, it appears that some wild-type coxIII protein is synthesized from the *cox3-1* AUA mutant transcript. In contrast, labeling of coxIII was completely eliminated (and respiratory growth abolished) by either a nuclear deletion mutation affecting another coxIII-mRNA-specific translational activator, *PET494*, or a mitochondrial *cox3* deletion mutation (Figure 5). While radiolabeling of cycloheximide-poisoned cells is not highly quantitative, densitometric scanning of autoradiograms indicated that the *cox3-1* mutation reduced coxIII synthesis roughly sevenfold. As a secondary effect (seen in some experiments, but not in all; unpublished results), the *cox3-1* mutation appeared to cause a relative increase in the labeling of ATPase subunit 6 (the band immediately below coxIII, which is partially obscured by coxIII in the wild-type lane 1, and less so in lane 6). While we do not know the reason for this increase, a similar increase in ATPase subunit 6 labeling was observed in the strain with the leaky *pet122* mutation (Figure 5), and has been seen in other mutants of the nuclear genes that code coxIII-mRNA-specific translational activators (COSTANZO, SEAVER and FOX 1986; FOX *et al.* 1988; MÜLLER *et al.* 1984). Although the apparent increase in the lower band of the coxIII/ATPase6 doublet is not unique to the *cox3-1* mutation, it remains possible that some of the increase in labeling at the position of ATPase subunit 6 in the *cox3-1* mutant corresponds to a shorter version of coxIII, aberrantly initiated downstream of the normal position (see DISCUSSION).

To facilitate subsequent analyses, we generated strains carrying the *rho*$^+$, *cox3-1* mitochondrial genome in the nuclear background isogenic to the standard wild-type D273-10B. The *cox3-1* diploid LSF20 was sporulated, and the mutant *rho*$^+$ genome of a resulting haploid was moved by cytoduction (CONDE and FINK 1976) and standard crosses into strains of the D273-10B background (LSF72 and LSF75; Table 1). The phenotypes of these strains were essentially

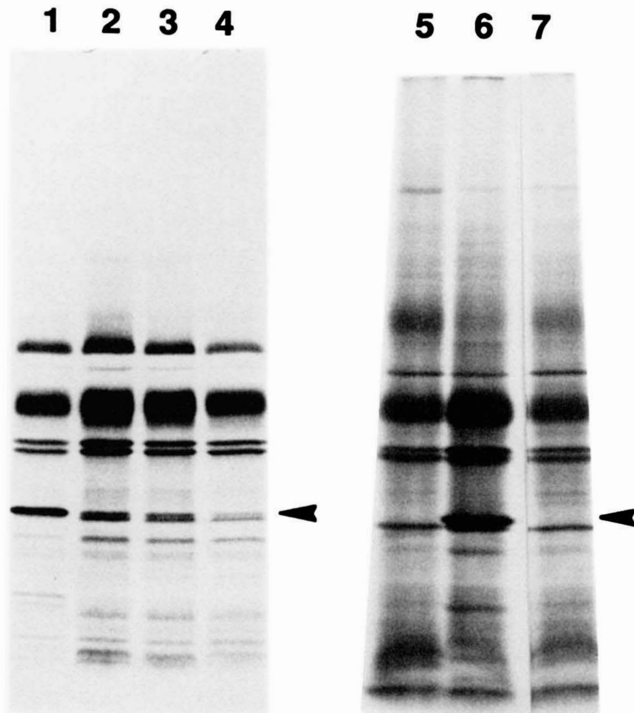

FIGURE 5.—Synthesis of coxIII protein is substantially reduced but not eliminated by the *cox3-1* mutation. Mitochondrial translation products were labeled *in vivo* as described in MATERIALS AND METHODS. Lane 1, the wild-type diploid, LSF18; lane 2, a *rho*$^+$, *cox3-1* strain derived by mating one of the two mitochondrial transformants to wild type (MATERIALS AND METHODS); lane 3, the *rho*$^+$, *cox3-1* diploid strain LSF20; lane 4, the leaky *pet122* (nuclear) mutant, TF175; lane 5, the *pet494* (nuclear) deletion mutant, MCC101; lane 6, wild-type, DL2; lane 7, a mitochondrial *cox3* deletion mutant, MCC118. Arrowheads show position of coxIII.

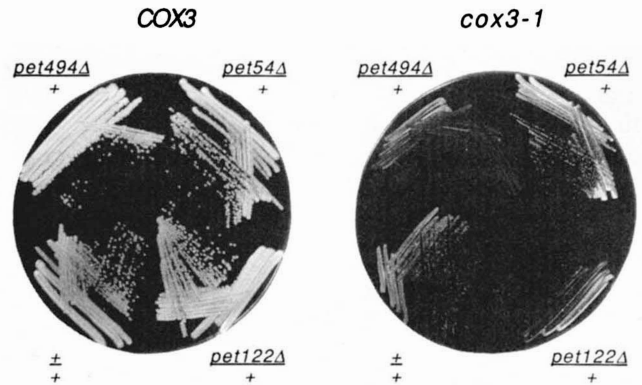

FIGURE 6.—*pet494* and *pet122* deletion mutations are fully recessive in diploids carrying wild-type mtDNA but not in diploids carrying the *cox3-1* mutation. Left, diploids with wild-type *COX3* mtDNA were streaked on YPEG and incubated at 30° for 3 days. Strains were: +/+, DUL1rho° × DAU2; *pet494*Δ/+, MCC101rho° × DUL1; *pet54*Δ/+, LSF97rho° × DAU2; *pet122*Δ/+, TWM10-41rho° × DUL1. Right, diploids with *cox3-1* mtDNA were streaked on YPEG and incubated at 30° for 5 days. Strains were: +/+, DUL1rho° × LSF72; *pet494*Δ/+, MCC101rho° × LSF75; *pet54*Δ/+, LSF97rho° × LSF72; *pet122*Δ/+, TWM10-41rho° × LSF75.

identical to LSF20 with respect to growth on YPEG medium and synthesis of coxIII (not shown).

To examine the effect of the initiation codon mutation on *COX3* mRNA levels, total RNA was isolated from three log phase cultures each of the *cox3-1* mutant strain LSF75 and an isogenic wild-type strain (DUL1) grown in YPGal. The RNAs were subjected to electrophoresis, blotted to a filter and hybridized to labeled probes for the *COX3* and actin mRNAs (MATERIALS AND METHODS). Hybridization signals were detected quantitatively with a Betascope 603 Blot Analyzer (Betagen Corp.). The steady-state level of *COX3* mRNA was not significantly affected by the *cox3-1* mutation: the level of *COX3* mRNA in the mutant, normalized to the internal standard of actin mRNA, was 1.2 (±0.4) times that of the wild type. Taken together with the respiratory growth phenotype and the *in vivo* protein labeling results, these findings indicate that the *cox3-1* mutation reduces translation of the message to a point where coxIII levels limit respiratory growth.

Translation of the mutant mRNA requires activity of *COX3*-specific translational activators: If residual translation of the mRNA bearing a mutant initiation codon represents reduced levels of a normal translation initiation process, then coxIII translation should still be dependent on the three mRNA-specific activators *PET494*, *PET54* and *PET122*. To examine this question, three diploids were constructed that carried the *cox3-1* mutation on mtDNA and were each heterozygous with wild type for a deletion mutation at one of the three nuclear genes (see legend to Figure 6). Upon sporulation, each of these diploids produced tetrads containing two progeny with the tight Pet$^-$ phenotype characteristic of the nuclear mutations and two with the leaky respiratory phenotype characteristic of the *cox3-1* mutation alone (6 tetrads for *pet494/PET494*, 8 for *pet54/PET54* and 7 for *pet122/PET122*). Thus, deletion mutations in the three *COX3*-mRNA-specific translational activators are all epistatic to *cox3-1*, indicating that residual translation initiation on the mutant mRNA requires the wild-type activation system.

While constructing the heterozygous diploids, we noticed that the *cox3-1* initiation codon mutation made the rate of respiratory growth abnormally sensitive to the intracellular levels of two of the three translational activators. In strains carrying wild-type mtDNA, *pet494*, *pet54* and *pet122* deletion alleles were fully recessive to wild-type, as judged by growth on YPEG medium (Figure 6, left panel). However, in strains carrying the *cox3-1* mitochondrial mutation, diploids heterozygous for a deletion of either *pet494* or *pet122*, but not *pet54*, grew more slowly than a homozygous wild-type isogenic strain (Figure 6, right panel). This synergistic effect may reflect an interaction between events occurring at the upstream site(s) of mRNA-specific translational activation (COSTANZO and FOX 1988) and events at the initiation codon

itself. It also suggests that the PET494 and PET122 proteins may be present at near limiting levels in wild-type cells. Interestingly, the absence of any effect in the *pet54* heterozygote is consistent with the observation that *PET54* is expressed at roughly ten times the level of *PET494* and *PET122* (MARYKWAS and FOX 1989; S. ZONGHOU, B. A. BARLOW and T. D. FOX, unpublished results).

DISCUSSION

As a first step toward studying the process of yeast mitochondrial translation initiation in detail, we have generated a site-directed mutation in the AUG codon previously identified (BROWNING and RAJBHANDARY 1982; THALENFELD and TZAGOLOFF 1980) as the initiator for coxIII translation. Insertion of this mutation, *cox3-1*, into an otherwise wild-type mitochondrial genome resulted in a leaky (partial) respiration-deficient phenotype, as judged by the ability of mutant cells to grow on nonfermentable carbon sources. As expected, this growth phenotype appears to be due to reduced translation of the *COX3* mRNA, since *in vivo* radiolabeling experiments demonstrated a specific reduction in the labeling of coxIII, while RNA-gel blot experiments revealed that the *cox3-1* mutation had little, if any, effect on the steady state level of the mRNA.

One of the most interesting features of this initiation codon mutation is that it does not completely block coxIII translation. Residual initiation could be occurring at the position of the mutant AUA initiator codon, at some other nearby codon, or perhaps at the next in-frame AUG in the structural gene, which is codon 16. We believe it likely that at least some residual initiation occurred at the mutant AUA codon, since the coxIII polypeptide synthesized in the mutant precisely comigrated both with the residual coxIII of a leaky nuclear mutation affecting coxIII translation and with wild-type coxIII during SDS-gel electrophoresis. Unfortunately, we have been unable to determine the initiation codons by Edman degradation of radiolabeled coxIII, presumably due to N-terminal blockage by f-*Met* (unpublished results). However, if all initiation in the *cox3-1* mutant were occurring at the next downstream AUG, then the resulting variant polypeptide, shorter by 15 amino acid residues, should have migrated detectably farther (approximately 1.5 mm) in the SDS-gel of Figure 6, and not at the position of wild-type coxIII. We cannot rule out the possibility that some downstream initiation did occur, yielding a product that comigrated with ATPase subunit 6 in the experiment of Figure 5. Nor can we exclude the possibility of aberrant initiation at some other nearby non-AUG codon. However, the N-terminal sequences of coxIII from human, *N. crassa* and *S. cerevisiae* are strongly conserved: the sequences are identical at the first two amino acids in all three species, at the first three residues in yeast and human, and at 16 of the first 23 residues in Neurospora and yeast (BROWNING and RAJBHANDARY 1982). Thus, there is a distinct possibility that a protein produced by downstream initiation, lacking 15 N-terminal residues, would not be functional.

How could the mutant AUA codon be recognized for initiation? By analogy with other systems (CIGAN, FENG and DONAHUE 1988; HARTZ, MCPHEETERS and GOLD 1989), the mitochondrial initiator $tRNA^{fMet}$ might recognize the AUA codon by wobble and help position initiation. Indeed, AUA serves as an initiation codon in animal mitochondria (reviewed in FOX 1987; KOZAK 1983). However, in animal mitochondria the initiation codon is usually at the immediate 5′-end of the mRNA, with little or no untranslated leader sequence preceding it. In contrast, the yeast *COX3* mRNA 5′-leader contains 98 AUA triplets and one AUG, making it difficult to see how recognition of AUA by the $tRNA^{fMet}$ *alone* could account for a significant level of starts at the mutant AUA initiation codon of the *cox3-1* message. Similarly, it appears highly unlikely that the *COX3*-mRNA-specific translational activators could specify the precise position of the initiation codon since their site(s) of action lie at least 172 bases upstream of the start codon in the wild-type mRNA and can be moved, with respect to the initiation codon, without loss of function (COSTANZO and FOX 1988; M. C. COSTANZO, unpublished results).

These considerations suggest that the site of translation initiation on the *COX3* mRNA is probably specified not only by the AUG codon, but also by some other feature of the sequence context (or structure) in that region. In *E. coli*, translation initiation sites are usually marked by Shine-Dalgarno sequences as well as other sequence information surrounding the initiation codon whose recognition mechanism is less well understood (GOLD 1988; SCHNEIDER *et al.* 1986; STORMO, SCHNEIDER and GOLD 1982). Interestingly, these sequences can be recognized *in vitro* by *E. coli* ribosomes even in the absence of any tRNA (HARTZ *et al.* 1991). However in yeast mitochondria, Shine-Dalgarno pairing between the 5′-mRNA-leader sequences and the 3′-end of the 15S rRNA does not appear to be generally important. Although short sequences complementary to the mitochondrial 15S rRNA 3′-domain can be found in most wild-type 5′-leaders, they occur at widely varied distances from the initiation codons (−8 to −107; LI *et al.* 1982), unlike the narrow range of distances required for a functional interaction in prokaryotes. Moreover, a chimeric mRNA that lacks these complementary se-

quences is translated *in vivo* (COSTANZO and FOX 1988).

Examination of the mRNA sequences surrounding the initiation codons of the eight major yeast mitochondrial mRNAs (DE ZAMAROCZY and BERNARDI 1986) reveals eight apparently conserved positions that could serve as guides for initiation site selection by the ribosome or some unknown factor. At positions −25, −13, −16, −3, +6, +15 and +18 (relative to the A of the AUG at +1, with no zero position) seven out of the eight mRNAs contain A, while at position +12 all eight contain A. The worst matches to this consensus are the coxIII and ATPase subunit 6 mRNAs, which each have A at six of the eight positions. The statistical significance of this observation is low (SCHNEIDER *et al.* 1986), owing to the very small number of mRNA sequences that can be compared and the fact that they have a highly biased base composition (approximately half the bases in the leaders are A, as are about 40% of the third position bases in codons). However, an examination of the sequences flanking 27 AUG triplets that do not start major translation products, taken from the 5′-leaders of these mRNAs, suggests that the consensus might be meaningful: only two of the non-start AUGs have flanking sequences that match at least six of the eight positions. In addition, the distribution of bases at each consensus position summed over these 27 sequences is random. Thus it is plausible to speculate that translation initiation sites are at least partially specified by sequences flanking the initiation codon, and that these sequences direct a low level of initiation on the *cox3-1* mutant mRNA. Clearly the biological significance of this flanking sequence consensus will have to be tested by mutational analysis.

Another feature of the seven mitochondrial mRNAs with long leaders that may also be worthy of note is that they contain U-rich tracts at variable distances upstream of the start codons. Recent evidence suggests that U-rich sequences may play a role in translation initiation by promoting both mRNA binding by *E. coli* ribosomes (BONI *et al.* 1991) and internal initiation on picornavirus RNA by eukaryotic cytoplasmic ribosomes (JACKSON, HOWELL and KAMINSKI 1990).

Whether the position of the *COX3* initiation codon is marked by the consensus described above or some other, yet to be elucidated, sequence or structure, its utilization is dependent upon mRNA-specific translational activators working through a site (or sites) upstream in the 5′-leader (COSTANZO and FOX 1986, 1988; COSTANZO, SEAVER and FOX 1986; FOX *et al.* 1988; KLOECKENER-GRUISSEM, MCEWEN and POYTON 1988; MÜLLER *et al.* 1984). Like normal translation of the wild-type mRNA, the residual translation of the *cox3-1* mutant mRNA is dependent upon these activator proteins, encoded by the nuclear genes *PET494* (COSTANZO *et al.* 1986), *PET54* (COSTANZO, SEAVER and FOX 1989) and *PET122* (HAFFTER, MCMULLIN and FOX 1990; OHMEN, KLOECKENER-GRUISSEM and MCEWEN 1988). Indeed, respiratory growth of *cox3-1* mutant strains is sensitive to decreased dosage of genes *PET494* and *PET122* in heterozygous mutant diploids, unlike the growth of strains carrying wild-type mtDNA (Figure 6). While the roles of the PET494 and PET54 proteins are unknown, the PET122 product has been shown genetically to interact functionally with protein components of the small subunit of mitochondrial ribosomes (HAFFTER, MCMULLIN and FOX 1990; HAFFTER, MCMULLIN and FOX 1991; MCMULLIN, HAFFTER and FOX 1990). Taken together, these findings suggest a model in which ribosomal small subunits first interact with the *COX3* mRNA in a reaction mediated by the mRNA-specific activators, and then identify the site of translation initiation by recognizing both the AUG codon and other local features. This model may prove generally correct for yeast mitochondria, since translation of at least four other mitochondrial mRNAs also requires, or appears to require, mRNA-specific activators (ACKERMAN *et al.* 1991; DECOSTER *et al.* 1990; DIECKMANN and TZAGOLOFF 1985; FOX *et al.* 1988; POUTRE and FOX 1987; RÖDEL 1986; RÖDEL and FOX 1987).

Finally, in this paper we have demonstrated the feasibility of using yeast mitochondrial transformation to achieve the replacement of wild type by mutant sequences *via* homologous recombination. In addition to producing the *cox3-1* point mutation, the strategy described here has been used in this laboratory to generate defined deletion mutations in *rho*$^+$ mtDNA as well as to insert nonmitochondrial sequences into a fully functional mitochondrial genome (M. C. COSTANZO, P. E. THORSNESS and T. D. FOX, unpublished results).

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