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In vivo analysis of mutated initiation codons in the mitochondrial *COX2* gene of *Saccharomyces cerevisiae* fused to the reporter gene *ARG8^m* reveals lack of downstream reinitiation

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Abstract To examine normal and aberrant translation initiation in *Saccharomyces cerevisiae* mitochondria, we fused the synthetic mitochondrial reporter gene *ARG8^m* to codon 91 of the *COX2* coding sequence and inserted the chimeric gene into mitochondrial DNA (mtDNA). Translation of the *cox2*(*1–91*)::*ARG8^m* mRNA yielded a fusion protein precursor that was processed to yield wild-type Arg8p. Thus mitochondrial translation could be monitored by the ability of mutant chimeric genes to complement a nuclear *arg8* mutation. As expected, translation of the *cox2*(*1–91*)::*ARG8^m* mRNA was dependent on the *COX2* mRNA-specific activator *PET111*. We tested the ability of six triplets to function as initiation codons in both the *cox2*(*1–91*)::*ARG8^m* reporter mRNA and the otherwise wild-type *COX2* mRNA. Substitution of AUC, CCC or AAA for the initiation codon abolished detectable translation of both mRNAs, even when *PET111* activity was increased. The failure of these mutant *cox2*(*1–91*)::*ARG8^m* genes to yield Arg8p demonstrates that initiation at downstream AUG codons, such as *COX2* codon 14, does not occur even when normal initiation is blocked. Three mutant triplets at the site of the initiation codon supported detectable translation, with efficiencies decreasing in the order GUG, AUU, AUA. Increased *PET111* activity enhanced initiation at AUU and AUA codons. Comparisons of expression, at the level of accumulated product, of *cox2*(*1–91*)::*ARG8^m* and *COX2* carrying these mutant initiation codons revealed that very low-efficiency translation can provide enough Cox2p to sustain significant respiratory growth, presumably because Cox2p is efficiently assembled into stable cytochrome oxidase complexes.

Key words *Saccharomyces cerevisiae* · Mitochondrial translation · Translation initiation · Downstream initiation

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Introduction

The mechanisms that control translation initiation in mitochondrial systems are poorly understood, owing to the deficiencies of organelle-derived in vitro translation systems (Dekker et al. 1993). However, it has been possible to ascertain some general features of translation initiation in fungal mitochondria by genetic analysis and examination of mRNA structure in *Saccharomyces cerevisiae* (reviewed in Fox 1996). While all of the eight ORFs encoding the major yeast mitochondrial translation products begin with AUG, it is not clear how, or how stringently, the initiation codon is selected. Six of the seven major mRNAs have long 5′-untranslated leaders (5′-UTLs) – 300 to 950 bases long – that contain AUG triplets upstream of their initiation codons (Grivell 1989; Dieckmann and Staples 1994), which argues against a scanning model similar to that for cytoplasmic translation (Sherman and Stewart 1982; Kozak 1989). Instead, it appears that mRNA sequence and structural information is probably employed to identify yeast mitochondrial initiation codons. This does not seem to involve a simple Shine-Dalgarno interaction, since sequences in the mRNAs that could pair in this manner with 15S rRNA (Li et al. 1982) can be mutationally destroyed without preventing translation (Costanzo and Fox 1988; Mittelmeier and Dieckmann 1995; Dunstan et al. 1997). Other possible interactions between mRNAs and rRNAs could play a role (Dunstan et al. 1997). Yeast mitochondrial translation depends on mRNA-specific translational activator proteins that

functionally interact with 5′-UTLs, and these activators could participate in initiation codon selection (reviewed in Fox 1996). Thus, the yeast mitochondrial initiation mechanism might be analogous to that of internal ribosome entry in eukaryotic cytoplasmic translation (Belsham and Sonenberg 1996; Kolupaeva et al. 1998).

Only very limited genetic analysis of mitochondrial initiation codons has been performed to date. AUG to AUA mutations have been created by site-directed mutagenesis and introduced in the *S. cerevisiae* genes *COX2* and *COX3*, which encode key subunits of cytochrome *c* oxidase (Folley and Fox 1991; Mulero and Fox 1994). In both cases, production of functional cytochrome oxidase subunits was strongly reduced but not eliminated. Synthesis of functional subunits indicated that at least some initiation occurred at the correct position. However, the occurrence of aberrant initiation at downstream AUG codons could not be ruled out, since this could have generated nonfunctional polypeptides that were undetectable in the previous studies. To remove this constraint we have here employed a reporter gene to monitor translation initiation, as has been done in bacterial and cytoplasmic systems (Zitomer et al. 1984; Clements et al. 1988; Gordon et al. 1992; Sacerdot et al. 1996; Sussman et al. 1996).

The nuclear gene *ARG8* encodes acetylornithine aminotransferase, an enzyme of the arginine biosynthetic pathway that is located in the mitochondrial matrix (Jauniaux et al. 1978; Heimberg et al. 1990). A synthetic, recoded, version of this gene, $ARG8^m$, can be phenotypically expressed as a reporter for mitochondrial gene expression when inserted into yeast mtDNA (Steele et al. 1996; He and Fox 1997). In this study, we have translationally fused $ARG8^m$ to an internal region of the *COX2* coding sequence, at codon 91, so that translation initiation, in its normal nucleotide context, could be monitored by the ability of the chimeric *cox2*(*1–91*)::$ARG8^m$ mitochondrial gene to complement the phenotype of a nuclear *arg8* mutation. Like translation of the native *COX2* mRNA (Poutre and Fox 1987; Mulero and Fox 1993b), translation of the *cox2*(*1–91*)::$ARG8^m$ mRNA depends specifically on the presence of the product of the nuclear gene *PET111*, which activates translation via functional interactions with the 54-nucleotide 5′-UTL of *COX2* mRNA (Mulero and Fox 1993a).

We have studied the ability of mutant initiation codons to allow expression of both the *cox2*(*1–91*)::$ARG8^m$ reporter and otherwise wild-type *COX2*. Some of the mutant initiation codons caused reduced accumulation of the *COX2* gene product, cytochrome oxidase subunit II (Cox2p), but allowed respiratory growth. These same mutations caused more severe reductions in accumulation of the *cox2*(*1–91*)::$ARG8^m$ reporter product, Arg8p, reflecting reduced translation initiation. Gene expression in these mutants was enhanced by overproduction of a hyperactive variant of the *COX2* mRNA-specific translational activator Pet111p. Other mutations abolished detectable translation of both *COX2* and *cox2*(*1–91*)::$ARG8^m$ mRNAs. Our results demonstrate that initiation at downstream AUG codons in the chimeric reporter mRNA does not occur detectably in this system, indicating stringent selection of the initiation site. This work provides new tools with which to search for *cis*- or *trans*-acting elements of the mitochondrial translation system that govern start site selection in this system.

Materials and methods

Yeast strains, media, genetic techniques and plasmids

All strains used in this study are listed in Table 1, with the exception of TF236 (see below). Strain NB40-3C is a spore obtained by "forced" sporulation (Fox et al. 1991) between strains DFS188 (Steele et al. 1996) and GW116 (Dunstan et al. 1997), which carries a 660-bp deletion in the upstream region of *COX2* (−295 to +363 relative to AUG, termed *cox2–62*). NB80 was created by cytoduction of wild-type mitochondria into a ρ° derivative of NB40-3C. NB151-1B was derived from the *pet111*::*LEU2* strain NB35-5D (unpublished) by "forced" sporulation. In the *pet111*::*LEU2* allele (C. A. Strick, unpublished) the 2-kb *Xba*I-*Bgl*II fragment of *PET111* has been replaced by a 2.7-kb *LEU2* fragment. TF236 (*MAT**a*** *lys2 leu2-3,112 ura3-52 ino1*Δ::*His3* arg8::*hisG pet9 ura3-52 lys2 his3? [cox3*::$arg8^m$*-1* ρ^+ *]*) was derived from a meiotic segregant of a cross between a D273-10B (ATCC25627) related strain and 777-3A (Weiss-Brummer et al. 1979). TF236 carries a $MnCl_2$-induced mutation, termed *cox3*::$arg8^m$*-1*, that maps to the $ARG8^m$ coding sequence and causes an Arg^- phenotype.

Fermentable complete medium was YPDA or YpGalA (1% yeast extract, 2% Bacto-peptone, 100 mg/l adenine, and either 2% glucose or 2% galactose supplemented with 0.1% glucose). Nonfermentable medium was YPEG (1% yeast extract, 2% Bacto-peptone, 100 mg/l adenine, 3% ethanol, 3% glycerol). Minimal medium (0.67% yeast nitrogen base without amino acids) was supplemented with 2% glucose and specific amino acids, uracil and adenine as required. Standard genetic methods were as described (Rose et al. 1988; Fox et al. 1991). Yeast nuclear transformation was carried out using the one-step transformation method (Chen et al. 1992), with plasmids YEp352 (Hill et al. 1986), pJM20 (Mulero and Fox 1994) and pJM57 (Mulero and Fox 1993a).

Construction of *cox2::*$ARG8^m$ fusion genes, and initiation codon mutagenesis

The pTZ18u (BioRad)-derived plasmid pHD6 (H. M. Dunstan, unpublished), which carries the $ARG8^m$ ORF fused to flanking *COX2* untranslated regions, was modified using a two step PCR strategy (Horton et al. 1989) to construct the *cox2*(*1–91*)::$ARG8^m$ translational fusion. The resulting plasmid contains the following elements: 0.57 kb of 5′-flanking mtDNA including the *COX2* promoter, the first 91 codons of *COX2* precisely fused to codon 2 of $ARG8^m$ (Fig. 1A), 0.81 kb of 3′-flanking mtDNA, and a 0.75-kb *Pac*I-*Mbo*I *COX3* mtDNA fragment that can rescue the *cox3-10* mutation (Costanzo and Fox 1993) following transformation. We eliminated the *Nsi*I site contained in the *COX3* fragment, so that the *Nsi*I site at position +65 of the *COX2* coding sequence would be unique. The final plasmid, pNB81, contains three unique sites useful for making further alterations: *Pac*I at −60, *Nsi*I at +65 and *Eag*I at +270 (all positions are given relative to the first base of the *COX2* initiation codon).

The *cox2*(*1–69*)::$ARG8^m$ translational fusion was constructed by replacing the 330-bp *Pac*I-*Eag*I fragment of pNB81 with a corresponding 273-bp *Pac*I-*Eag*I PCR product containing the first 69 *COX2* codons and the modified codons 90 and 91 from the *cox2*(*1–91*)::$ARG8^m$ fusion (Fig. 1A).

Mutations in the *COX2* initiation codon were generated by replacing the wild-type *COX2* *Pac*I-*Nsi*I fragment with mutant *Pac*I-*Nsi*I fragments created by two-step PCR (Ho et al. 1989)

Table 1 Strains used in this study

Strain[a]	Nuclear genotype	Mitochondrial genotype	Mutant initiation codon	Source
Isogenic to D273-10B (ATCC25627)				
NB80	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	COX2 ρ^+		This study
HMD22	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2::ARG8*^{m} ρ^+		This study
NB53	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2(1–69)::ARG8*^{m} ρ^+		This study
NB43	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2(1–91)::ARG8*^{m} ρ^+		This study
NB60	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-10* ρ^+	AUA	This study
NB110	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-10(1–91)::ARG8*^{m} ρ^+	AUA	This study
NB160	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-30* ρ^+	CCC	This study
NB83	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-30(1–91)::ARG8*^{m} ρ	CCC	This study
NB161	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-31* ρ^+	GUG	This study
NB131	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-31(1–91)::ARG8*^{m} ρ^+	GUG	This study
NB162	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-32* ρ^+	AAA	This study
NB132	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-32(1–91)::ARG8*^{m} ρ^+	AAA	This study
NB163	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-33* ρ^+	AUC	This study
NB133	*MAT**a** lys2 leu2–3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-33(1–91)::ARG8*^{m} ρ^+	AUC	This study
NB164	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-34* ρ^+	AUU	This study
NB134	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-34(1–91)::ARG8*^{m} ρ^+	AUU	This study
NB97	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-60* ρ^+		This study
NB54	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-60(1–91)::ARG8*^{m} ρ^+		This study
NB40-3C	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG*	*cox2-62* ρ^+		This study
NB40-3Cρ°	*MAT**a** lys2 leu2–3,112 ura3–52 his3ΔHinDIII arg8::hisG*	ρ°		This study
DL2	*MAT**a** lys2*	*COX2* ρ^+		Folley and Fox (1991)
HMD107	*MAT**a** lys2*	*cox2-107* ρ^+		Dunstan et al. (1997)
MCC125	*MAT**a** lys2*	*cox3-10* ρ^+		Costanzo and Fox (1993)
NB151-1B	*MAT**a** pet111::LEU2 lys2 ura3-52 his3ΔHinDIII arg8::hisG leu2?*	*cox2(1–91)::ARG8*^{m}		This study
Isogenic to DBY947 (Neff et al. 1983)				
NB46	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2(1–69)::ARG8*^{m} ρ^+		This study
NB169	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2-31* ρ^+	GUG	This study
NB104	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2-60* ρ^+		This study
NB66	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2-60(1–91)::ARG8*^{m} ρ^+		This study
NB41	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2-62* ρ^+		This study
NB63	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox2-107* ρ^+		This study
NB71	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*cox3::arg8*^{m}-1 ρ^+		This study
NB62	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	*COX2* ρ^- (*pPT25*)		This study
DFS160ρ	*MATα leu2Δ arg8ΔURA3 ura3-52 kar1-1 ade2-101*	ρ°		Steele et al. (1996)

[a] For the genotype and derivation of strain TF236, see Materials and methods

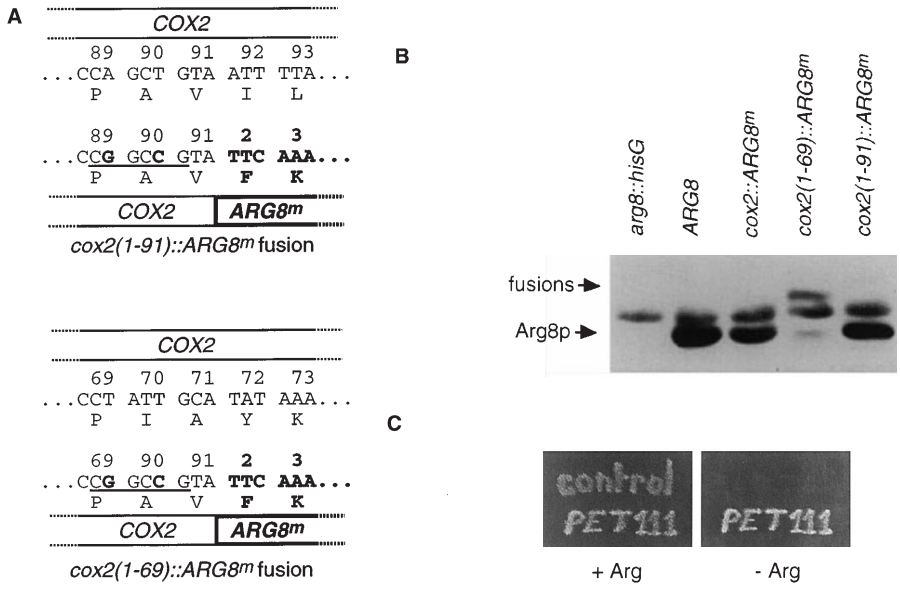

Fig. 1A–C Structure, expression and *PET111* dependence of *cox2*::*ARG8*^{m} chimeric genes inserted into yeast mtDNA. **A** Sequences of junctions between *COX2* and *ARG8*^{m}. *Numbers* refer to *COX2* or *ARG8*^{m} codons. *Bold letters and numbers* are used to indicate *ARG8*^{m} sequences as well as alterations in the *COX2* sequence. The *Eag*I restriction site created by the silent mutations in *COX2* is *underlined* (see Materials and methods for details of construction). **B** Accumulation of fusion proteins and mature Arg8p resulting from mitochondrial expression of chimeric *cox2*::*ARG8*^{m} genes. 50 μg of total cellular protein from strains NB40-3C (Δarg8), DL2 (*ARG8*), HMD22 (*cox2*::*ARG8*^{m}), NB53 [*cox2*(*1–69*)::*ARG8*^{m}] and NB43 [*cox2*(*1–91*)::*ARG8*^{m}] were subjected to SDS gel electrophoresis and Western analysis with anti-Arg8p antiserum. The positions of mature Arg8p and fusion proteins are indicated. A fusion protein from the *cox2*(*1–91*)::*ARG8*^{m} strain is detectable upon longer exposure of the blot (not shown). The middle band corresponds to a cross-reacting protein which is present in *arg8* deletion strains. **C** Phenotypic expression of *cox2*(*1–91*)::*ARG8*^{m} is dependent on *PET111*. The *cox2*(*1–91*)::*ARG8*^{m} *arg8 pet111-9* strain NB151-1B was transformed with either an empty vector Yep352 ("control") or the *PET111*-carrying plasmid pJM20 ("PET111") (see Materials and methods). The transformants were regrown and then replica-plated to either minimal medium containing arginine (+Arg) or minimal medium lacking arginine (−Arg), and incubated for 2 days at 28° C

using the high-fidelity polymerase Deep Vent (New England Biolabs). To place mutations in the initiation codon of *cox2*(*1–91*)::*ARG8*^{m}, we used pNB90, a derivative of pNB81 carrying the mutation *cox2-22* (Bonnefoy and Fox, in preparation), which introduces a unique *Sna*BI restriction site between the *Pac*I and *Nsi*I sites. Thus cloning efficiency of mutant fragments could be improved by digestion of the ligation reaction with *Sna*BI. To place mutations in the *COX2* gene itself, the pJM2 (Mulero and Fox 1993a) *Pac*I site upstream of *COX2* was rendered unique by shortening the *COX2* 3′-flanking region, creating pNB69, and the *cox2-22* mutation was introduced in pNB69 to yield pNB82, which was then used for cloning of mutated fragments as described above for pNB90.

To facilitate the insertion of mutant alleles into mtDNA, we constructed a deletion mutation, *cox2-60*, that removed nucleotides −63 to +66 relative to the *COX2* initiation codon, by deleting the *Pac*I-*Nsi*I fragment from both *COX2* (pNB69) and *cox2*(*1–91*)::*ARG8*^{m} (pNB81).

Plasmids containing fusion genes and mutations were checked by DNA sequencing using mainly the primer (COX2A) complementary to positions −254 to −237 relative to the *COX2* initiation codon.

Mitochondria transformation

The plasmids carrying modified *COX2* sequences were introduced into the ρ° mitochondria of strain DFS160ρ° (Table 1) by high-velocity microprojectile bombardment, as previously described (Wiesenberger et al. 1995), together with the *LEU2* plasmid YEp351 (Hill et al. 1986) to select nuclear transformants. To identify transformants, colonies were mated to strains carrying mutations in either *ARG8*^{m} (TF236), *COX3* (MCC125) or *COX2* (HMD107), as appropriate. Following mating, cells were replica-plated to minimal medium lacking arginine or to nonfermentable medium as appropriate, to detect mitochondrial transformants by their ability to rescue the tester mutations. Mutant mtDNA sequences from purified mitochondrial transformants were integrated into ρ^+ mitochondrial genomes by cytoduction (Fox et al. 1991) into strains carrying either the *cox2-60* deletion (NB54 or NB97) or the *cox2-62* deletion (Dunstan et al. 1997) (NB40-3C) in the *COX2* 5′ region. The desired cytoductants were detected by their ability to rescue the *cox2-107* mutation (Dunstan et al. 1997) in strain NB63. Crosses to strains NB62 (ρ^- *COX2*) and NB71 (*cox3*::*arg8*^{m}*-1*) were performed to confirm the ρ^+ state of the cytoductants and the presence of the *ARG8*^{m} ORF as appropriate. In two cases, we constructed the desired mitochondrial genome by directly bombarding a ρ^+ strain (Johnston et al. 1988) harboring a deletion in the *COX2* 5′ region (NB41 or NB104) with mutant mtDNA plasmids and YEp351. The ρ^+ mitochondrial transformants were directly selected by replica-plating the Leu$^+$ transformants to nonfermentable medium (to construct the *cox2-31* strain NB169) or onto medium lacking arginine (to construct the *cox2*(*1–69*)::*ARG8*^{m} strain NB46). The ρ^+ genomes from NB169 and NB46 were then transferred to NB40-3ρ° by cytoduction, to yield strains NB161 and NB53.

All mitochondrial mutations were verified by DNA sequencing following integration into ρ^+ mtDNA. Yeast genomic DNA (Hoffman and Winston 1987) was used as a template to amplify the *COX2* region around the AUG (corresponding to positions −315 to +266 relative to the initiating AUG) by PCR. The PCR product was sequenced directly with primer COX2A (above). All sequencing reactions were carried out by the Synthesis and Sequencing Facility in the Cornell Biotechnology Building.

Analysis of cellular RNA and protein

Total RNA was prepared and analyzed as described (Dunstan et al. 1997), except that samples were supplemented with ethidium bromide before application to the gels for electrophoresis. The *COX2* and *ARG8*^{m} probes were a mixture of the 1.6-kb *Pac*I

fragment from pJM2 (Mulero and Fox 1993a), which contains the whole *COX2* coding region and some flanking sequences, and the 1.4-kb *Pac*I-*Bam*HI fragment from pNB81, which contains part of the *COX2* gene (approximately from the start of the mature mRNA to codon 91) and the whole $ARG8^m$ coding sequence. The control 15S probe was the 2-kb *Bam*HI fragment from plasmid pYJL25 (Tian et al. 1991), provided by O. Groudinsky.

Steady-state levels of Cox2p and Arg8p were analyzed by Western blotting of total proteins (Yaffe 1991) fractionated on 12% or 10% polyacrylamide gels, respectively, and probed with monoclonal anti-Cox2p (dilution 1/5000, Molecular Probes, provided by G. Dujardin) and polyclonal anti-Arg8p (Steele et al. 1996) (dilution 1/1000) antibodies. Secondary anti-mouse or anti-rabbit antibodies were detected using the Pierce chemiluminescent substrate.

Results

Translational fusion of the mitochondrial reporter gene $ARG8^m$ to *COX2*

To analyze the effect of mutations in the *COX2* initiation codon on translation, using a growth phenotype that is independent of the function of Cox2p, we inserted translational fusions of *COX2* to the synthetic reporter gene $ARG8^m$ (Steele et al. 1996) into mtDNA. *COX2* specifies a precursor protein with a 15-amino acid leader peptide that is processed to yield the mature protein (Sevarino and Poyton 1980; Pratje and Guiard 1986). Our fusions contained the beginning of the pre-Cox2p coding region, to preserve the normal *COX2* mRNA initiation codon context. Furthermore, the reporter portion of the chimeric genes comprised the entire coding sequence of pre-Arg8p (except for its initiation codon), which is normally imported from the cytoplasm under the direction of its N-terminal signal targeting sequence. Thus, we expected the mitochondrially synthesized reporter protein to be cleaved by the matrix processing protease, releasing the normal mature Arg8p in its normal cellular location, the mitochondrial matrix (Steele et al. 1996). This would ensure that nonstandard initiation events in the *COX2* portion of the chimeric mRNAs could neither alter the structure nor diminish the activity of the reporter protein.

We constructed two chimeric genes, specifying fusion proteins with the pre-Arg8p moiety fused to pre-Cox2p after the 69th or the 91st pre-Cox2p amino acid (Fig. 1A). These constructs were transformed into mitochondria (Materials and methods) and integrated in place of *COX2* in mtDNA of a strain carrying a disrupted *arg8*::*hisG* allele at the nuclear *ARG8* locus. Expression of both chimeric genes, *cox2*(*1–69*)::$ARG8^m$ and *cox2*(*1–91*)::$ARG8^m$, in mtDNA fully complemented the nuclear *arg8*::*hisG* mutation when cells were grown on medium lacking arginine (not shown). Western analysis using anti-Arg8p antibody showed that both fusion proteins were expressed, but that cleavage of the pre-Arg8p processing site was incomplete, especially in the case of the *cox2*(*1–69*)::$ARG8^m$ product (Fig. 1B). We therefore employed the *cox2*(*1–91*)::$ARG8^m$ reporter gene for all subsequent studies.

As a first step, we verified that expression of the chimeric *cox2*(*1–91*)::$ARG8^m$ gene was dependent on the *COX2* mRNA-specific translational activator Pet111p. As expected, the *pet111*, *arg8*, *cox2*(*1–91*)::$ARG8^m$ strain NB151-1B failed to grow on medium lacking arginine, but arginine prototrophy was restored by transformation of this strain with a plasmid carrying the wild-type *PET111* gene (Fig. 1C) and by crossing it to the *PET111 arg8* ρ° strain DFS160ρ° (data not shown). Thus, the reporter exhibits normal dependence on translational activation.

Residual initiation at some mutant codons allows accumulation of Cox2p

In addition to the previously described AUA mutation (Mulero and Fox 1994), we constructed five new initiation codon mutations by site-directed mutagenesis of plasmids carrying either the *COX2* gene or the *cox2*(*1–91*)::$ARG8^m$ fusion. These six mutations included all three possible modifications of the last nucleotide, the GUG triplet, and alterations involving two (AAA) or three (CCC) nucleotide positions in the initiation codon.

We first examined the phenotypes caused by these mutations when introduced into the *COX2* gene of otherwise wild-type strains (Materials and methods). Their cytochrome spectra all exhibited the typical absorption peaks of cytochromes *c*, *c1* and *b*. However, the mutants carrying initiation codons AUC, AAA and CCC, lacked a detectable peak of cytochrome *aa3*, corresponding to cytochrome *c* oxidase (Fig. 2). The cytochrome *aa3* absorption peak was identical to wild type in the mutant with a GUG initiation codon, was slightly but reproducibly decreased in the AUU mutant, and was very low in the AUA mutant.

Analysis of respiratory growth on nonfermentable medium yielded a similar ranking amongst the mutants (Fig. 3, top panel). The GUG mutant was almost indistinguishable from the wild type. Growth of the AUU mutant was only slightly weaker, and could only be differentiated from that of the GUG mutant by streaking or by a brief incubation of patches printed to nonfermentable medium. The AUA mutant grew poorly compared to wild type, as previously described (Mulero and Fox 1994). Finally, the triplets CCC, AAA and AUC completely abolished respiratory growth. Immunodetection of steady-state levels of Cox2p in the mutants and control strains (Fig. 3, top panel) showed that the pattern of protein accumulation closely mirrored the growth and cytochrome spectra in all cases, as expected.

All mutations reduce translation of reporter mRNA

Respiratory growth phenotypes of the mutants reflect translation of the *COX2* mRNA, insertion of newly synthesized pre-Cox2p into the inner membrane, processing of the leader-peptide, and assembly into the stable cytochrome *c* oxidase complex. To focus more

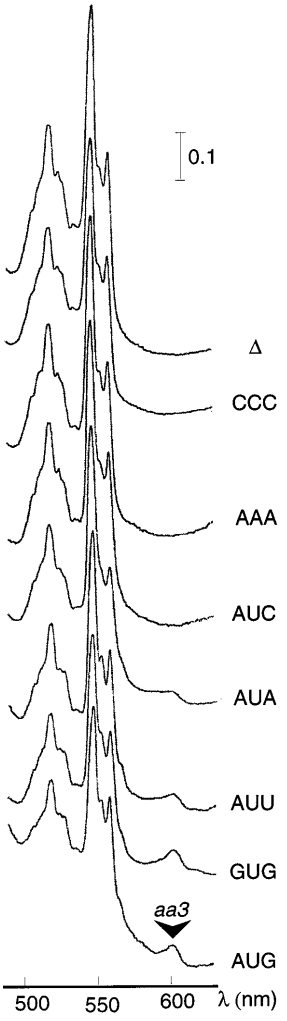

Fig. 2 Whole-cell cytochrome absorption spectra of strains harboring either wild-type or mutant initiation codons in the *COX2* gene. Low temperature absorption spectra of dithionite-reduced cytochromes from galactose-grown cells were recorded in liquid nitrogen as described (Claisse et al. 1970). The absorption maximum expected for the alpha band of cytochrome *aa3* at 602 nm is indicated. The absorption peaks at 558, 552 and 546 nm correspond to the alpha bands of cytochromes *b*, *c1* and *c*, respectively. The absorbance scale is indicated at the *top right*. The strains examined were: Δ, NB97; CCC, NB160; AAA, NB162; AUC, NB163; AUA, NB60; AUU, NB164; GUG, NB161; AUG, NB80

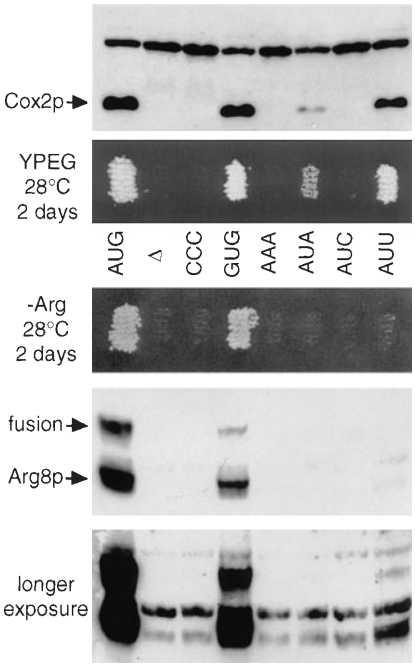

Fig. 3 Effects of initiation codon mutations in *COX2* and *cox2(1–91)::ARG8^m* on growth phenotypes and protein accumulation. The panels at the *top* show data for strains carrying the *COX2* gene, while the *bottom* panels show data for strains carrying the *cox2(1–91)::ARG8^m* reporter. Cells were patched onto complete glucose medium, replica-plated onto selective medium (top, nonfermentable; bottom, minimal medium lacking arginine) and incubated for 2 days at 28° C. Relative growth patterns were similar at 16° C and 36° C, and after 7 days of incubation, except that the AUU mutant with *cox2(1–91)::ARG8^m* (strain NB134) showed slight growth after incubation for 5–7 days on minimal medium lacking arginine, especially at 36° C (not shown). For Western analysis, 100 μg or 150 μg of total cellular protein from each strain was fractionated by electrophoresis on SDS gels and reacted with anti-Cox2p or anti-Arg8p antibodies, respectively (Materials and methods). Overexposure of the blot probed with anti-Arg8p (*lowest panel*) revealed the presence of Arg8p initiated by the AUU codon, in addition to cross-reacting proteins present in the *arg8* deletion strain. The strains examined, carrying *COX2* or *cox2(1–91)::ARG8^m*, respectively, were: AUG, NB80 and NB43; Δ, NB97 and NB54; CCC, NB160 and NB83; GUG, NB161 and NB131; AAA, NB162 and NB132; AUA, NB60 and NB110; AUC, NB163 and NB133; AUU, NB164 and NB134

closely on translation, we introduced the six initiation codon mutations into the plasmid-borne *cox2(1–91)::ARG8^m* reporter fusion, whose product is a soluble enzyme. These mutant reporter genes were then integrated into the mitochondrial genome, in place of *COX2*, such that all surrounding regulatory sequences would then be identical to those of wild-type *COX2* (Materials and methods). As expected from the above results, the GUG initiation codon allowed substantial growth on medium lacking arginine (Fig. 3). However, the AUU initiation codon led to only very weak growth – detectable after a 5- to 7-day incubation – that was enhanced at 36° C (not shown). No Arg^+ growth was detected for the AUA mutant.

In agreement with these growth phenotypes, Western analysis using an anti-Arg8p antibody to detect steady-state accumulation of Arg8p showed that while the GUG mutant contained a significant level of the reporter protein, it was still substantially reduced relative to wild-type (Fig. 3). The AUU mutant contained very low levels of Arg8p, requiring overexposure for detection (Fig. 3). Arg8p was undetectable in the other mutants (AUA, CCC, AAA and AUC) above the background level of a crossreacting protein present in the *arg8* deletion control.

The contrast between the respiratory and Arg phenotypes of the respective AUA and AUU mutants raised the possibility that extraneous mutations in the mitochondrial or nuclear genomes of the *cox2(1–91)::ARG8^m*

strains could be responsible for the Arg$^-$ phenotypes. To exclude this possibility we crossed all of the strains used in the experiment shown in Fig. 3 with NB66 (Table 1), whose mtDNA contains a 129-bp deletion that removes the region including the *COX2* initiation codon, upstream of the reporter [*cox2-60*(*1–91*)::*ARG8*^{m}, Materials and methods]. The resulting zygotes thus contained a second copy of all mitochondrial and nuclear sequences, except the deleted region. Diploids were selected on appropriate minimal medium containing arginine, allowing mtDNA recombination to occur, and then replica-plated to minimal medium lacking arginine. In every case, the Arg phenotype of the diploids was the same as that of the corresponding haploid initiation codon mutants, confirming that no extraneous mutations were affecting this phenotype.

The steady-state level of *COX2* mRNA harboring an AUA initiation codon mutation is very similar to that of the wild-type *COX2* mRNA (Mulero and Fox 1994). To ask whether the steady-state levels of the mutant chimeric *cox2*(*1–91*)::*ARG8*^{m} mRNAs were also similar to wild type, we carried out Northern analysis on total cellular RNA from each strain, using a *COX2-ARG8*^{m} probe (Fig. 4). Quantitation of the results by PhosphoImager (Molecular Dynamics) detection revealed that the levels of *cox2*(*1–91*)::*ARG8*^{m} mRNA were similar to or higher than wild-type in all the mutants, except for the one containing a CCC initiation codon, which expressed 60% of the wild-type level. Thus, the Arg$^-$ phenotypes of the mutants were not due to defects in mRNA stability, but rather to decreased translation of the chimeric mRNA. In addition, the steady-state level of chimeric *cox2*(*1–91*)::*ARG8*^{m} mRNA was roughly similar to that of the normal *COX2* mRNA.

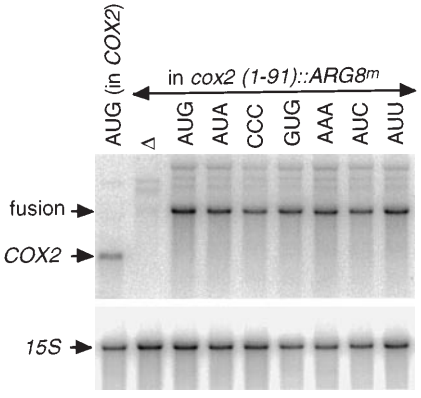

Fig. 4 The chimeric *cox2*(*1–91*)::*ARG8*^{m} mRNA accumulates despite the presence of mutant initiation codons. Total cellular RNA from the strains NB80 (AUG *COX2*), NB54 (Δ), NB43 (AUG), NB110 (AUA), NB83 (CCC), NB131 (GUG), NB132 (AAA), NB133 (AUC), NB134 (AUU) was subjected to electrophoresis and then hybridized to a mixture of *COX2* and *ARG8*^{m} probes (see Materials and methods), revealing either the wild-type *COX2* mRNA, or the *cox2*(*1–91*)::*ARG8*^{m} RNA (fusion), depending on the strain. The same blot was then hybridized to a probe for the 15S rRNA. Hybridization was quantified using a PhosphoImager (Molecular Dynamics)

Multicopy suppression of initiation codon mutations

Increased gene dosage of the *COX2* mRNA-specific translational activator gene, *PET111*, was previously shown to enhance translation of a *COX2* mRNA bearing an AUA initiation codon (Mulero and Fox 1994). We therefore tested the whether elevated *PET111* activity could suppress all the initiation codon mutations in both a *COX2* and *cox2*(*1–91*)::*ARG8*^{m} context, as a more sensitive way to detect low levels of translation initiation. To maximize possible suppression effects, we employed a multicopy plasmid bearing the dominant *PET111-20* allele. *PET111-20* has a missense mutation that suppresses some mutations in the *COX2* mRNA 5′-UTL (Mulero and Fox 1993a; A. Seshan and T. D. Fox, unpublished results), and the AUG to AUA initiation codon mutation (Mulero and Fox 1993a). Thus, *PET111-20* appears to encode a more active product than the wild-type allele.

We transformed the mutant strains with a control vector or a multicopy plasmid carrying *PET111-20*, and examined the respiratory growth or arginine prototrophy of the transformants, as appropriate (Fig. 5). In the context of the *COX2* gene, both the AUA and AUU mutations were clearly suppressed, as expected. In the context of the *cox2*(*1–91*)::*ARG8*^{m} reporter, the AUU mutation exhibited clear suppression, while the AUA mutation was only weakly suppressed (detectable after prolonged incubation). The AUC, AAA and CCC mutations were not detectably suppressed by elevated *PET111* activity, even after prolonged incubation, suggesting that translation of these mRNAs is either completely blocked or extremely weak.

Discussion

In this study we have examined in vivo the ability of altered *COX2* initiation codons to function in mito-

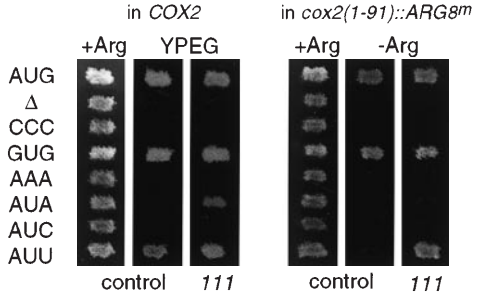

Fig. 5 Analysis of multicopy suppression of the initiation codon mutants by the gene encoding the *COX2* translational activator Pet111p. Strains carrying either *COX2* or the *cox2*(*1–91::ARG8*^{m} reporter, as indicated (see legend to Fig. 3 for strain names), were transformed with the empty vector Yep352 (control) (Hill et al. 1986), or the *PET111-20* carrying plasmid pJM57 (*111*), (Mulero and Fox 1993a). Transformants were patched onto arginine-containing minimal medium selective for the *URA3* plasmid marker (+Arg), and then replica-plated to either nonfermentable medium (YPEG), or minimal medium lacking arginine (−Arg), as appropriate. Cells were incubated at 28° C for 2 days

chondrial synthesis of the normal gene product, Cox2p, and a soluble reporter enzyme, Arg8p, that is released by a processing protease from a chimeric Cox2p-Arg8p fusion protein. The use of this reporter system should have allowed the detection of translation initiation events at downstream AUG codons in *COX2*, such as the one at position 14, since the aberrant initiation events would not affect the structure or stability of the reporter protein, whose coding sequence begins at codon 92. A previous study examined the effects of an AUG to AUA *COX2* initiation codon mutation and showed that the detectable residual *COX2* translation was initiated at the mutant AUA triplet, rather than the AUG at position 14 (Mulero and Fox 1994). However functional constraints on the structure of pre-Cox2p could have prevented detection of additional downstream initiation events. The fact that the various initiation codon mutations either reduced or completely abolished all detectable synthesis of the reporter protein demonstrates, for the first time, that downstream translation starts do not occur, even when normal initiation is blocked. Thus, yeast mitochondrial initiation site selection is under stringent control, at least for the *COX2* mRNA.

How might the mitochondrial translation machinery discriminate between the initiation codon position and the AUG codon at position 14? A previously suggested sequence consensus surrounding yeast mitochondrial initiation codons (A residues at positions −25, −13, −6. +6, +12, +15 and +18; Folley and Fox 1991) does not appear to play a role here, since it is present around both positions. We favor the hypothesis that the translational activator Pet111p, or proteins associated with it, interact with the mitochondrial ribosome and direct it to the initiation codon. In this model, the initiation site would be determined by the geometry of the functional interaction been Pet111p and the *COX2* mRNA 5′-UTL (Mulero and Fox 1993a). Direct interactions between the ribosome and the 5′-UTL (Green-Willms et al. 1998) could also play a role here. Subsequent translation initiation would then depend upon a productive interaction between a codon in the selected region and the anticodon of the initiator tRNA.

Three mutant triplets at the initiation codon position supported detectable translation, with efficiencies decreasing in the order GUG, AUU, AUA. The comparison between the effects of each mutation on *COX2* and *cox2*(*1–91*)::*ARG8*^{m} expression revealed that accumulation of Cox2p in functional cytochrome *c* oxidase complexes was less affected than accumulation of the soluble reporter protein Arg8p. For example, the AUG to AUU mutation in *COX2* had only a modest effect on respiratory growth and the steady state level of Cox2p, while the same mutation in *cox2*(*1–91*)::*ARG8*^{m} greatly diminished growth in the absence of arginine as well as the level of Arg8p. These differences are not due to dramatic alterations in the steady-state-levels of the *COX2* and *cox2*(*1–91*)::*ARG8*^{m} mRNAs, nor are they likely to be due to differences in the efficiencies of translation initiation since the mRNA 5′-UTL and initiation codon context are the same in both mRNAs. We propose instead that the differences observed between Cox2p and the reporter protein reflect post-translational differences in the behavior of the proteins.

Mitochondria contain ATP-dependent proteolytic systems that play a role in assembly of membrane complexes and degradation of unassembled mitochondrial translation products (reviewed in Rep and Grivell 1996). Indeed, unassembled Cox2p is known to be a target of such proteolysis through the action of Yme1p (Nakai et al. 1995; Pearce and Sherman 1995; Leonhard et al. 1996; Weber et al. 1996). Thus, Cox2p produced in excess of the amount needed to assemble with other cytochrome *c* oxidase subunits would be quickly degraded. On the other hand, if Cox2p synthesis were reduced below this level, then all of the available Cox2p would be fully assembled into stable complexes. Thus, the stability of Cox2p could depend on its rate of synthesis, limiting the range over which its accumulation could vary. In contrast, the soluble reporter protein Arg8p does not assemble with other polypeptides and is likely to have a constant half-life that is independent of its rate of synthesis. Indeed, Arg8p can accumulate to levels far higher than wild-type when it is overproduced (Steele et al. 1996). These considerations suggest that while respiratory growth is the most sensitive way to detect low levels of *COX2* mRNA translation, Arg8p accumulation more accurately reflects relative rates of synthesis. We therefore conclude, for example, that initiation at GUG is several times less efficient than at AUG, based on the relative levels of Arg8p, despite the fact that Cox2p accumulation in the corresponding mutant was essentially wild-type.

Our results support the idea that there could be genes in yeast mtDNA whose ORFs initiate with GUG, AUU or AUA. At least one such gene has been previously proposed (Colin et al. 1985), but there is no experimental evidence to support this suggestion. Vertebrate mitochondrial translation systems employ alternative start codons such as AUA and AUU in humans (Fearnley and Walker 1987) and GUG in ducks (Pan et al. 1993) and fish (Johansen et al. 1990). GUG also appears to be utilized for initiation in mitochondria of filamentous fungi (Netzker et al. 1982) and plants (Bock et al. 1994; Siculella et al. 1996; Sakamoto et al. 1997), as well as in chloroplasts (Rochaix et al. 1989; Turmel et al. 1993). Recently, the *COX2* mRNA from radish was shown to have an ACG initiation codon, which is apparently not edited to AUG (Dong et al. 1998). However, an ACG mutation at the human *COX2* initiation codon apparently prevents translation and is associated with a mitochondrial encephalomyopathy (Clark et al. 1999). Interestingly, mitochondrial translation systems in nematodes may not employ AUG at all, utilizing UUG, AUU, AUA and GUU instead (Okimoto et al. 1990). In bacterial and eukaryotic cytoplasmic systems, non-AUG codons occur naturally in some mRNAs and can play a role in controlling gene

expression (Hager and Rabinowitz 1985; Sacerdot et al. 1996; Arnaud et al. 1999, and references therein).

Mutational analyses of initiation codons in *E. coli* genes (Sacerdot et al. 1996; Sussman et al. 1996) and eukaryotic nuclear genes of yeast (Zitomer et al. 1984; Clements et al. 1988; Donahue and Cigan 1988), plants (Gordon et al. 1992) and animals (Mehdi et al. 1990; Kozak 1997), as well as *Chlamydomonas* chloroplast genes (Chen et al. 1993, 1995), have shown that a number of non-AUG codons can direct detectable but, in most cases, very low levels of, translation initiation. The identification of suppressors of initiation codon mutations has helped to elucidate the function of several factors that control initiation specificity in bacteria (Sussman et al. 1996) and the yeast cytoplasmic system (Yoon and Donahue 1992; Huang et al. 1997). Similar analyses have begun in organellar systems, but no specific factors have been identified to date (Folley and Fox 1994; Chen et al. 1997). The identification of nonfunctional initiation codon mutations in this study will aid in the extension of this genetic approach to yeast mitochondria. The AUC, AAA and CCC triplets failed to direct detectable translation, even when we elevated the activity of the translational activator gene *PET111*, a condition that enhanced translation initiation at partially functional codons. Thus, these alleles should provide an excellent starting point for the selection of strong *cis*- and *trans*-acting suppressor mutations whose analysis may provide insights into the mechanism of start site selection in yeast mitochondria.

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