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Accumulation of mitochondrially synthesized *Saccharomyces cerevisiae* Cox2p and Cox3p depends on targeting information in untranslated portions of their mRNAs

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The essential products of the yeast mitochondrial translation system are seven hydrophobic membrane proteins and Var1p, a hydrophilic protein in the small ribosomal subunit. Translation of the membrane proteins depends on nuclearly encoded, mRNA-specific translational activators that recognize the 5′-untranslated leaders of their target mRNAs. These translational activators are themselves membrane associated and could therefore tether translation to the inner membrane. In this study, we tested whether chimeric mRNAs with the untranslated sequences normally present on the mRNA encoding soluble Var1p, can direct functional expression of coding sequences specifying the integral membrane proteins Cox2p and Cox3p. DNA sequences specifying these chimeric mRNAs were inserted into mtDNA at the *VAR1* locus and expressed in strains containing a nuclearly localized plasmid that supplies a functional form of Var1p, imported from the cytoplasm. Although cells expressing these chimeric mRNAs actively synthesized both membrane proteins, they were severely deficient in cytochrome *c* oxidase activity and in the accumulation of Cox2p and Cox3p, respectively. These data strongly support the physiological importance of interactions between membrane-bound mRNA-specific translational activators and the native 5′-untranslated leaders of the *COX2* and *COX3* mRNAs for localizing productive synthesis of Cox2p and Cox3p to the inner membrane.

Keywords: membrane targeting/mitochondrial translation/mRNA localization/mRNA-specific translational activation

Introduction

The targeting of membrane proteins synthesized in the well-studied bacterial and eukaryotic cytoplasmic systems has typically been found to depend on signals contained within the proteins themselves or their precursors (Na *et al*., 1992; Corsi and Schekman, 1996; Rapoport *et al*., 1996; Schatz and Dobberstein, 1996; Wickner and Leonard, 1996; Neupert, 1997; Saavedra *et al*., 1997). Within mitochondria, the signals and mechanisms that target membrane proteins encoded by mtDNA have not been studied directly, owing to the lack of *in vitro* protein synthesis systems derived from the organelles.

Genetic analysis in *Saccharomyces cerevisiae* has demonstrated that translation of several mitochondrially coded mRNAs depends on membrane-bound mRNA-specific translational activators that recognize the 5′-untranslated leaders (UTLs) of their target mRNAs (Fox, 1996). These activator proteins, encoded by nuclear genes, appear to mediate the productive interaction between the mRNAs and mitochondrial ribosomes at the surface of the inner membrane. They are required for the translation of both normal mitochondrial mRNAs (Fox, 1996), and chimeric mRNAs specifying the soluble mitochondrial reporter-protein Arg8^{m}p (Steele *et al*., 1996; N.S.Green-Willms and T.D.Fox, unpublished data). Taken together, these studies have suggested that membrane insertion of mitochondrially coded proteins such as cytochrome *c* oxidase subunits II and III (Cox2p and Cox3p), as well as cytochrome *b*, could depend on this system to tether mitochondrial translational initiation complexes to the membrane (Costanzo and Fox, 1990; Maleszka *et al*., 1991; Michaelis *et al*., 1991; McMullin and Fox, 1993; Fox, 1996). Such a system could prevent mislocalized translation of proteins destined for the inner membrane. However, unlike the analogous SRP/SRP-receptor system of eukaryotic cytoplasms (Walter and Lingappa, 1986; Ng and Walter, 1994), this model has the interesting and novel implication that information contained in the yeast mitochondrial mRNA 5′-UTLs should be important for membrane localization of the proteins encoded by those mRNAs.

There are eight major translation products specified by yeast mtDNA, seven of which are hydrophobic subunits of energy-transducing enzyme complexes located in the inner mitochondrial membrane (Tzagoloff and Myers, 1986). However, one major yeast mitochondrial gene product, termed Var1p, is a hydrophilic ribosomal protein in the mitochondrial small ribosomal subunit (Groot *et al*., 1979; Terpstra and Butow, 1979; Terpstra *et al*., 1979; Hudspeth *et al*., 1982). Whereas nothing is presently known about what, if any, specific factors may be required for activation of *VAR1* mRNA translation, it is clear from the exceptional nature of the polypeptide and its ultimate destination, that its synthesis should not depend upon a mechanism devoted to membrane protein insertion. Thus, the 5′-UTL of the *VAR1* mRNA should not contain information for the localized translation of membrane proteins.

In this study, we have taken advantage of our ability to relocate synthetic yeast mitochondrial and nuclear genes (Sanchirico *et al*., 1995; Steele *et al*., 1996) to ask, *in vivo*, whether any targeting information for mitochondrially coded membrane proteins is contained in the untranslated

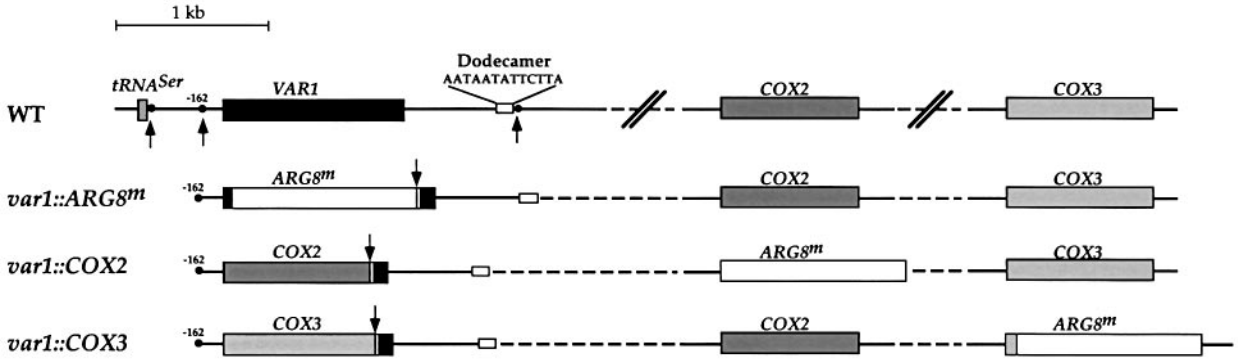

Fig. 1. Schematic representation of the mitochondrial DNA from strains expressing the coding sequences of *ARG8*^{m}, *COX2* or *COX3* from *VAR1*. The coding sequences are indicated as follows: *VAR1* (black box), *COX2* (dark grey box), *COX3* (light grey box), *ARG8*^{m} (open box). The mature *VAR1* transcript begins at bp –162 (black circle) (Smooker *et al*., 1988) and ends at the conserved dodecamer sequence (small open box). The *var1::ARG8*^{m} was constructed such that the first 17 codons from *VAR1*, followed by three codons generated by insertion of an *Acc*I site, are fused in-frame at the start codon for the *ARG8*^{m} structural gene. The *var1::COX2* and *var1::COX3* 5′-end gene fusions occur exactly at the *VAR1* ATG codon, and the fusion junctions at the 3′ ends of the *COX2* and *COX3* ORFs immediately downstream of the normal *COX2* and *COX3* stop codons are out of frame with respect to the 166-bp segment of the *VAR1* ORF (see Methods and materials). The arrows in the *var1::ARG8*^{m}, *var1::COX2* and *var1::COX3* chimeric genes indicate the positions of the termination codons used in the respective ORFs. Thus, the Cox2p and Cox3p proteins expressed from the chimeric genes are identical to wild-type. The construction of the *cox3::ARG8*^{m} gene fusion was described previously (Steele *et al*., 1996). The *cox2::ARG8*^{m} construct is a precise fusion of the *ARG8*^{m} initiation codon to the corresponding position of *COX2*: it contains no *COX2*-coding sequence (H.M. Dunstan and T.D.Fox, unpublished data).

portions of their mRNAs. By inserting the *COX2* and *COX3* coding sequences into chimeric mRNAs containing the 5′- and 3′-untranslated regions (UTRs) of the *VAR1* mRNA, we tested whether the *VAR1* untranslated sequences, normally present on a ribosomal protein mRNA, can direct functional expression of coding sequences specifying the integral membrane proteins Cox2p and Cox3p. DNA sequences specifying the chimeric mRNAs were inserted into mtDNA and expressed in strains containing a nuclearly localized plasmid that supplies, *in trans*, a functional form of Var1p imported from the cytoplasm (Sanchirico *et al*., 1995). Our results demonstrate that although both membrane proteins were translated from the chimeric mRNAs, their incorporation into active cytochrome *c* oxidase complexes was severely defective. These data strongly support the physiological importance of interactions between membrane-bound, mRNA-specific translational activators and the native 5′-UTLs of the *COX2* and *COX3* mRNAs for localizing productive synthesis of Cox2p and Cox3p to the inner membrane.

Results

Mitochondrial transformation and integration of the var1::ARG8^m*, var1::COX2 and var1::COX3 chimeric genes into the mitochondrial genome*

The *var1::ARG8*^{m}, *var1::COX2* and *var1::COX3* genes were designed to place the functional expression of the synthetic mitochondrial reporter gene *ARG8*^{m} (Steele *et al*., 1996), or *COX2* or *COX3*, respectively, under *VAR1* transcriptional and translational control (Figure 1; Materials and methods). It is important to note that the *var1::COX2* and *var1::COX3* chimeric genes specify wild-type Cox2p and Cox3p, respectively. As a first step towards integrating these constructs into mtDNA, synthetic *rho*$^-$ strains, each carrying the *var1::ARG8*^{m}, *var1::COX2* or the *var1::COX3* chimeric genes, were obtained by mitochondrial transformation of the *rho*^{0} strain DFS160 with plasmids pMES7, pMES18 or pMES21, respectively (Materials and methods). Mitochondrial transformants carrying the *var1::ARG8*^{m} gene were identified by mating to the *arg8 rho*$^+$ strain DFS188 containing the *VAR1*^{u} expression vector, pEVA1, and selecting for Arg$^+$ diploids. Only synthetic *rho*$^-$ mitochondrial transformants carrying the *var1::ARG8*^{m} sequence are capable of forming Arg$^+$ diploids in this mating assay. Mitochondrial transformants carrying either *var1::COX2* or *var1::COX3* were identified by mating to the *cox2* mutant strain TF192 or the *cox3* mutant strain LSF5, respectively. Only synthetic *rho*$^-$ mitochondrial transformants carrying *COX2* or *COX3* sequences are capable of forming respiratory competent (Pet$^+$) diploids in these mating assays. Stable synthetic *rho*$^-$ strains were isolated through repeated rounds of subcloning and testing in the mating assays.

Next, we isolated a *rho*$^+$ diploid strain in which the *VAR1* coding sequence was replaced by *ARG8*^{m}. To do this, the synthetic *rho*$^-$ transformant MSY362 (Table I) carrying the *var1::ARG8*^{m} construct was crossed to the *rho*$^+$ strain DFS188 (Table I), which contained the *VAR1*^{u} expression plasmid pEVA1 in its nucleus, such that mitochondrial translation could be maintained in the absence of the mitochondrial *VAR1* gene. As expected, this cross yielded a respiratory competent Arg$^+$ diploid, MSY573 (Table I), indicating that the *ARG8*^{m} gene was expressed functionally from the *var1::ARG8*^{m} chimeric locus and that *VAR1*^{u} covered the function of the deleted *VAR1* coding sequence. Both respiratory growth and Arg$^+$ prototrophy were lost in mitotic segregants of MSY573 that lacked the nuclear *VAR1*^{u} expression plasmid pEVA1, confirming that the mitochondrial *VAR1* gene had been inactivated. Southern analysis confirmed the replacement of *VAR1* by *var1::ARG8*^{m} in the mtDNA of this diploid (data not shown).

To study expression of *COX2* from the *VAR1* locus, we generated a diploid in which *VAR1* was replaced by *var1::COX2*, and *COX2* was replaced by *cox2::ARG8*^{m}. This diploid, MSY575 (Table I), was made by mating the

Table I. Strains used in this study

Strain	Genotype	Reference
DFS188	*MAT***a** *leu2-3,-112 his-ΔHin*dIII *lys2 ura3-52 arg8::hisG* [D273–10B *rho*$^+$]	Steele *et al.* (1996)
DFS160rho°	*MAT*α *leu2-3,-112 ura3-52 ade2-101 arg8Δ::URA3 gal2 kar1-1* [*rho*^{0}]	Steele *et al.* (1996)
TF192	*MAT***a** *lys2 ade1* [*rho*$^+$ *cox2-V25*]	this study
LSF5	*MAT***a** *lys2 opi1* [*rho*$^+$ *cox3-M7583*]	this study
MSY362	*MAT*α *leu2-3,-112 ura3-52 ade2-101 arg8Δ::URA3 gal2 kar1-1* [pMES7 *rho*$^-$]	this study
MSY493	*MAT*α *leu2-3,-112 ura3-52 ade2-101 arg8Δ::URA3 gal2 kar1-1* [pMES18 *rho*$^-$]	this study
MSY523	*MAT*α *leu2-3,-112 ura3-52 ade2-101 arg8Δ::URA3 gal2 kar1-1* [pMES21 *rho*$^-$]	this study
HMD22	*MAT***a** *leu2-3,-112 his3-ΔHin*dIII *lys2 ura3-52 arg8::hisG* [D273–10B *rho*$^+$ *cox2::ARG8*^{m}]	Steele *et al.* (1996)
DFS189	*MAT***a** *leu2-3,-112 his3-ΔHin*dIII *lys2 ura3-52 arg8::hisG* [D273–10B *rho*$^+$ *cox3::ARG8*^{m}]	this study
MCC60R2-16	*MAT***a** *cbs1::TRP1 ade2 his3-Δ1 leu2-3,-112* [*rho*$^+$ *MSUcbs1-2*]	Costanzo and Fox (1988)
MCC62	MATα *cbs1::TRP1 his3Δ1* [*rho*^{0}]	Costanzo and Fox (1988)
JJM194	*MAT*α *ade2-101 ura3-52 pet111–11 pet494–2* [pJM30 *rho*$^-$]	Mulero and Fox (1993b)
JJM195	*MAT*α *ade2-101 ura3-52 pet111–11 pet494–2* [pJM41 *rho*$^-$]	Mulero and Fox (1993b)
JJM102	*MAT***a** *ade2-101 ura3-52 pet111–11* [*rho*$^+$]	Mulero and Fox (1993b)
PTH44	*MAT***a** *ade2-101 ura3-52 pet494–2* [*rho*$^+$]	Mulero and Fox (1993b)
MSY509	*MAT***a**/α *leu2-3,-112/leu2-3,-112 ura3-52/ura3-52 +/ade2-101 arg8::hisG/arg8Δ::URA3 +/kar1-1 lys2/+ his3-ΔHin*dIII/+ [*rho*$^+$ *cox3:ARG8*^{m}] [pEVA1]	this study
MSY510	*MAT***a**/α *leu2-3,-112/leu2-3,-112 ura3-52/ura3-52 +/ade2-101 arg8::hisG/arg8Δ::URA3 +/kar1-1 lys2/+ his3-ΔHin*dIII/+ [*rho*$^+$ *cox2::ARG8*^{m}] [pEVA1]	this study
MSY573	*MAT***a**/α *leu2-3,-112/leu2-3,-112 ura3-52/ura3-52 +/ade2-101 arg8::hisG/arg8Δ::URA3 +/kar1-1 lys2/+ his3-ΔHin*dIII/+ [*rho*$^+$ *var1::ARG8*^{m}] [pEVA1]	this study
MSY575	*MAT***a**/α *leu2-3,-112/leu2-3,-112 ura3-52/ura3-52 +/ade2-101 arg8::hisG/arg8Δ::URA3 +/kar1-1 lys2/+ his3-ΔHin*dIII/+ [*rho*$^+$ *cox2::ARG8*^{m} *var1::COX2*] [pEVA1]	this study
MSY577	*MAT***a**/α *leu2-3,-112/leu2-3,-112 ura3-52/ura3-52 +/ade2-101 arg8::hisG/arg8Δ::URA3 +/kar1-1 lys2/+ his3-ΔHin*dIII/+ [*rho*$^+$ *cox3:ARG8*^{m} *var1::COX3*] [*pEVA1*]	this study

synthetic *rho*$^-$ transformant MSY493 (Table I) carrying the *var1::COX2* construct, with the *rho*$^+$, *cox2::ARG8*^{m} strain HMD22 (Table I), carrying the *VAR1*^{u} expression plasmid. It was capable of slow respiratory growth, indicating that the *var1::COX2* construct was partially functional and Arg$^+$. Replacement of *VAR1* by *var1::COX2* was confirmed by the dependence of both Arg$^+$ prototrophy and slow respiratory growth in the presence of the *VAR1*^{u} expression plasmid, and by Southern analysis of mtDNA (not shown).

A similar diploid, MSY577 (Table I), in which *VAR1* was replaced by *var1::COX3*, and *COX3* was replaced by *cox3::ARG8*^{m}, was generated by mating the synthetic *rho*$^-$ transformant MSY523 (Table I) with the *rho*$^+$, *cox3::ARG8*^{m} strain DFS189 (Table I), carrying the *VAR1*^{u} expression plasmid. This diploid also exhibited slow respiratory growth and Arg$^+$ prototrophy, both of which were *VAR1*^{u}-dependent. Southern analysis confirmed the replacement of *VAR1* by *var1::COX3* (not shown).

Synthesis of Cox2p and Cox3p

As a prerequisite to asking whether Cox2p or Cox3p could be stably incorporated into the inner membrane when translated from chimeric mRNAs bearing the *VAR1* 5′- and 3′-UTRs, we first determined whether these chimeric mRNAs were efficiently translated *in vivo*. To do this, we compared the pulse-labeling of mitochondrial gene products in the *var1::ARG8*^{m} strain (MSY573), containing wild-type *COX2* and *COX3* loci, with the *var1::COX2* strain (MSY575) and the *var1::COX3* strain (MSY577). Cells were grown to mid-exponential phase in complete medium containing galactose (YPGal), and mitochondrial translation products were pulse-labeled for 5 and 10 min *in vivo* with [^{35}S]methionine, following the addition of cycloheximide to prevent cytoplasmic protein synthesis (Materials and methods). The labeled mitochondrial translation products were analyzed by SDS–PAGE of samples containing equal amounts (80 μg) of mitochondrial protein, followed by phosphoimager scanning of the dried gel (Figure 2). This experiment demonstrated that the *var1::COX2* chimeric mRNA was translated at roughly the same

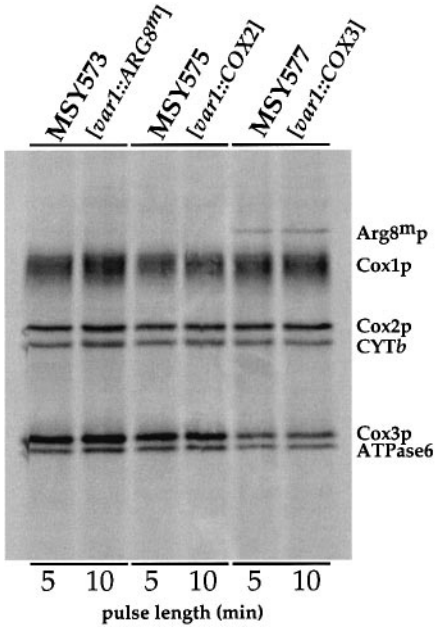

Fig. 2. Cox2p and Cox3p are labeled efficiently *in vivo* during short pulses when translated from *var1::COX2* and *var1::COX3* chimeric mRNAs, respectively. Strains MSY573 (*var1::ARG8m*), MSY575 (*var1::COX2*) and MSY577 (*var1::COX3*) were grown to mid-exponential phase in YPGal medium at 30°C. Mitochondrial translation products were pulse-labeled *in vivo* with [^{35}S]methionine in the presence of cycloheximide. Cells were labeled during pulses of 5 and 10 min, as indicated. Mitochondrial proteins (80 μg per lane) were resolved by SDS–PAGE in a 12.5% gel, and the dried gel was exposed to a phosphoimager screen. The mitochondrially encoded polypeptides are indicated on the right of the computer image from the phosphoimager scan.

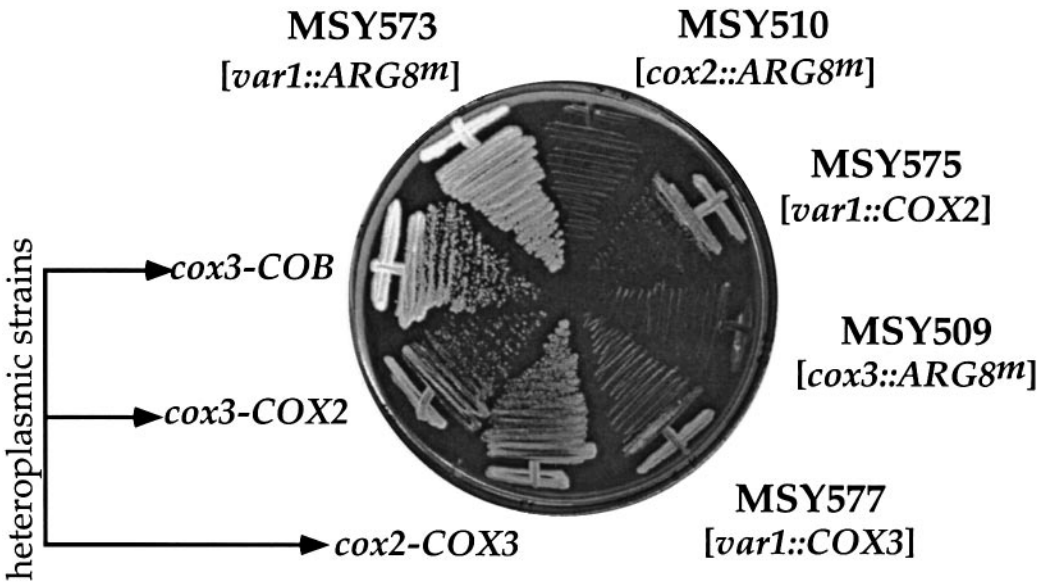

Fig. 3. Respiratory growth is reduced in strains expressing *var1::COX2* and *var1::COX3* chimeric mRNAs. Isonuclear diploid strains (Table I) MSY573 [*rho*$^+$ *var1::ARG8m*], MSY510 [*rho*$^+$ *cox2::ARG8*^{m}], MSY575 [*rho*$^+$ *cox2::ARG8*^{m} *var1::COX2*], MSY509 [*rho*$^+$ *cox3::ARG8*^{m}] and MSY577 [*rho*$^+$ *cox3::ARG8*^{m} *var1::COX3*] were streaked on YPGE agar, and incubated for 4 days at 30°C. Also streaked, were strains carrying the indicated chimeric genes heteroplasmically with wild-type mtDNA, in nuclear backgrounds that demanded expression of the chimeric genes (Costanzo and Fox, 1988; Mulero and Fox, 1993b): JJM194×JJM102 [*rho*$^+$, *rho*$^-$ *cox3-COX2*], JJM195×PTH44 [*rho*$^+$, *rho*$^-$ *cox2-COX3*] and MCC60R2-16×MCC62 [*rho*$^+$, *rho*$^-$ *cox3-COB*].

rate as the wild-type *COX2* mRNA. Translation of the *var1::COX3* mRNA was also robust, but appeared to be lower than that of the wild-type *COX3* mRNA by ~3-fold, as determined by phosphoimager quantitation of labeling after 5 min. However, there was little increase in Cox3p labeling after 5 min, suggesting that the protein translated from the *var1::COX3* mRNA could be highly unstable. In any event, the untranslated regions of the *VAR1* mRNA are clearly capable of directing translation of sequences coding the membrane proteins Cox2p and Cox3p.

The synthesis of mature Cox2p requires N-terminal cleavage of a larger precursor by the IMP protease complex (reviewed in He and Fox, 1997). This processing step requires translocation of the N-terminus of pre-Cox2p across the inner membrane. It is interesting, therefore, that radiolabeled Cox2p has the same electrophoretic mobility in each of the three strains examined in Figure 2, indicating that Cox2p expressed in the *var1::COX2* strain (MSY575) is apparently processed correctly. This result suggests that translation of pre-Cox2p from the *var1::COX2* chimeric mRNA does not prevent transport of its N-terminus through the inner membrane.

Another interesting aspect of the results shown in Figure 2 is that newly synthesized Arg8^{m}p is only observed in the *var1::COX3* strain. All of the strains examined in Figure 2 are phenotypically Arg$^+$ and therefore express the *ARG8*^{m} reporter gene, and although longer exposures show the presence of labeled Arg8^{m}p in all three strains, there is clearly differential labeling of Arg8^{m}p translated from the three different chimeric mRNA species. At present, we do not understand the basis for these differences, but it may be relevant that the N-terminal residues of the altered pre-Arg8p primary translation product are not identical (Figure 1; Methods and materials). Whereas matrix processing of these pre-proteins should yield the same mature product, it could be that either synthesis of the primary translation products or the kinetics of their processing and stability are different.

Respiratory phenotypes of var1::COX2 and var1::COX3 strains

Next, we examined the ability of strains expressing Cox2p or Cox3p from coding sequences located at the *VAR1* locus to grow on nonfermentable carbon sources. When streaked on medium containing only the carbon sources glycerol and ethanol, the strains bearing either *var1::COX2* or *var1::COX3* exhibited a slow-growth phenotype relative to the control strain bearing *var1::ARG8*^{m} and wild-type *COX2* and *COX3* genes (Figure 3). Whereas these slow-growth phenotypes indicated that functional expression of the *var1::COX2* and *var1::COX3* chimeric genes was defective relative to wild-type, they were clearly distinct from the completely nonrespiratory null phenotypes of *cox2::ARG8*^{m} and *cox3::ARG8*^{m} control strains. As expected, the slow growth on nonfermentable medium was accompanied by reduced rates of cellular respiration. The polarographically measured rates of cyanide-sensitive oxygen consumption were ~85% lower in the *var1::COX2* and *var1::COX3* strains compared with the *var1::ARG8*^{m} control strain (Table II).

Dramatically reduced respiratory growth is not a general consequence of expressing cytochrome *c* oxidase subunits from chimeric mRNAs. Strains that express these membrane proteins from chimeric genes that specify *cox2::COX3* or *cox3::COX2* mRNAs, carried in an unstable heteroplasmic state together with wild-type mtDNA (Mulero and Fox, 1993b), exhibited vigorous respiratory growth, as did a similar strain expressing a *cox3::COB* mRNA (Costanzo and Fox, 1988) (Figure 3). In each of these cases, the chimeric mRNAs bear the 5′-UTLs known to interact with membrane-bound, mRNA-specific

Table II. Mutant respiratory activities

Strain	Cyanide-sensitive respiration[a,b] (%)	Cytochrome *c* oxidase activity[b,c] (%)
MSY573	(100)	(100)
MSY575	16.2 ± 4.2	12.2 ± 3.5
MSY577	12.6 ± 2.3	7.9 ± 0.6

[a]Average of three experiments.
[b]Rates of cyanide-sensitive respiration in whole cells were determined polarographically as described in Materials and methods.
[c]Specific activities of cytochrome *c* oxidase in isolated mitochondria were determined spectrophotometrically as the rate of oxidation of reduced cytochrome *c*.

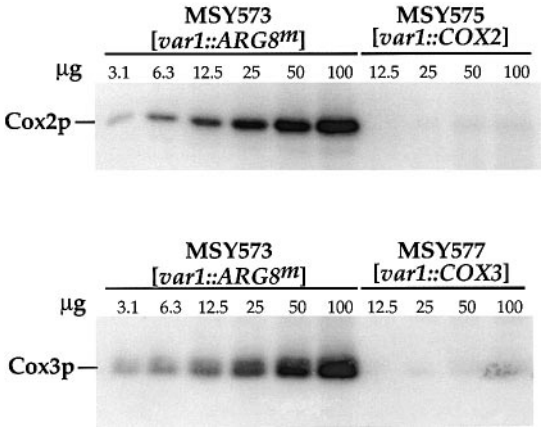

Fig. 4. The steady-state levels of Cox2p and Cox3p are severely reduced after translation of *var1::COX2* and *var1::COX3* chimeric mRNAs, respectively. Strains MSY573 (*var1::ARG8m*), MSY575 (*var1::COX2*) and MSY577 (*var1::COX3*) were grown to mid-logarithmic phase in YPGal at 30°C. Mitochondria were isolated and the indicated amounts of total mitochondrial protein were separated on a SDS–PAGE gel (12.5%), transferred to nitrocellulose, and incubated with monoclonal antibodies to either Cox2p (upper panel) or Cox3p (lower panel). The immune complexes were decorated with [^{125}I]anti-mouse immunoglobulin and the blots were exposed to a phosphoimager screen. The figure shows the computer-generated image of the phosphoimager scan. The relative signal intensities per microgram protein were: Cox2p; MSY573, 17.2 ± 1.9, MSY577, 0.7 ± 0.2 and Cox3p; MSY573, 15.7 ± 1.7, MSY577, 0.8 ± 0.3.

translational activators, in contrast to the *var1::COX2* and *var1::COX3* mRNAs.

Reduced Cox2p and Cox3p levels, and cytochrome c oxidase activity in mutant mitochondria

To determine the effects of *var1::COX2* and *var1::COX3* expression on cytochrome *c* oxidase activity and the accumulation of Cox2p and Cox3p, we performed enzyme activity assays and immunoblot analysis with mitochondria purified from MSY573, MSY575 and MSY577 cells grown in complete medium containing galactose. The specific activities of cytochrome *c* oxidase in mitochondria isolated from the *var1::COX2* (MSY575) and *var1::COX3* (MSY577) mutants were, respectively, 12.6 and 7.9% of the activity in the mitochondria from the control strain MSY573 (Table II). Moreover, the quantitative immunoblot analysis shown in Figure 4 indicated that the levels of Cox2p and Cox3p in MSY575 and MSY577 mitochondria are, respectively, ~5% of the amounts detected in the mitochondria from MSY573. Thus, the respiration-deficient phenotype of these strains appears to be caused by decreased levels of Cox2p or Cox3p when they are expressed from chimeric mRNAs bearing the untranslated regions of the *VAR1* mRNA. Since, as shown above, translation of the *var1::COX2* mRNA was essentially normal whereas translation of the *var1::COX3* mRNA was reduced only 3-fold, we conclude that decreased protein stability, not reduced translation of the chimeric mRNAs, is the major factor responsible for the low steady-state levels of Cox2p and Cox3p in the mutants.

Discussion

The key function of mitochondrial genetic systems is to promote respiration in eukaryotic cells by providing a few hydrophobic protein subunits of energy-transducing complexes in the inner membrane (Attardi and Schatz, 1988). This fact suggests that mitochondrial genetic systems are specialized for the synthesis of integral membrane polypeptides. If so, these organellar systems should possess features that localize translation of proteins on the surface of the inner membrane.

One interesting feature of the *S.cerevisiae* mitochondrial genetic system is that translation of at least five of the seven mitochondrial mRNAs encoding membrane proteins depends on nuclearly encoded mRNA-specific translational activator proteins (Fox, 1996). For example, translation of the *COX2* mRNA is specifically activated by the Pet111p nuclear gene product (Poutre and Fox, 1987; Mulero and Fox, 1993b), whereas translation of *COX3* mRNA is specifically activated by a complex containing the nuclear gene products of Pet54p, Pet122p and Pet494p (Müller *et al*., 1984; Costanzo and Fox, 1986; Costanzo *et al*., 1986; Fox *et al*., 1988a; Brown *et al*., 1994). In both cases, translation of the mRNAs is limited by the levels of their respective activators (Steele *et al*., 1996; N.S.Green-Willms and T.D.Fox, unpublished data). These translational activator proteins interact functionally with sites in the 5′-UTLs of their target mRNAs (Costanzo and Fox, 1988, 1993; Mulero and Fox, 1993a,b; Wiesenberger *et al*., 1995; Dunstan *et al*., 1997). In the case of the *COX3* mRNA-specific activator Pet122p, a functional interaction with mitochondrial ribosomes has also been demonstrated (Haffter *et al*., 1990, 1991; McMullin *et al*., 1990). Pet122p is an integral inner mitochondrial membrane protein (McMullin and Fox, 1993; C.A.Butler and T.D.Fox, unpublished data), as is the *COX2* mRNA-specific activator Pet111p (N.S.Green-Willms and T.D.Fox, unpublished data). Thus, in addition to their regulatory role, these activator proteins could tether translation initiation complexes involving the mRNAs encoding Cox2p and Cox3p to the mitochondrial inner membrane, by virtue of their interactions with the 5′-untranslated mRNA leaders. This model predicts that targeting information for mitochondrially synthesized proteins would reside, at least in part, in the leaders of the mRNAs that encode them.

The ribosomal protein Var1p is the only major yeast mitochondrial translation product that is not a hydrophobic membrane protein, and it is widely assumed, although not actually demonstrated, that Var1p is translated on free ribosomes in the matrix. Thus, the 5′-UTL of the *VAR1* mRNA is unlikely to interact with a membrane-bound,

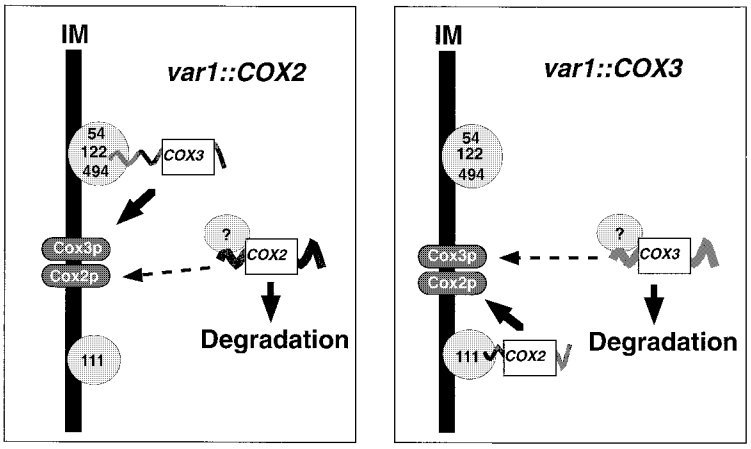

Fig. 5. Model for expression of the *var1::COX2* and *var1::COX3* chimeric mRNAs in yeast mitochondria. The UTRs of the *VAR1* mRNA (thick wavy lines) direct mislocalized translational initiation of either the *COX2* or *COX3* coding sequences, possibly activated by an unknown protein on free ribosomes in the matrix. Only a small proportion of the Cox2p or Cox3p translated in this way is assembled into cytochrome *c* oxidase in the membrane (dotted arrow), whereas the bulk of these proteins are degraded. Translation of the wild-type *COX2* mRNA is activated at the surface of the inner membrane (IM) by interaction of the activator protein Pet111p (111) with the *COX2* mRNA 5′-UTL (thin wavy line). Membrane-bound translation of the wild-type *COX3* mRNA is similarly activated by the Pet54p–Pet122p–Pet494p complex (54, 122, 494).

mRNA-specific, translational activator. Based on this premise, we asked whether the untranslated portions of the *VAR1* mRNA could promote the functional expression *in vivo* of *COX2* and *COX3* coding sequences in chimeric mRNAs. If membrane-bound, mRNA-specific translational activators are physiologically important for efficient, productive insertion of Cox2p and Cox3p in the inner membrane, then we expected that phenotypic expression of the *var1::COX2* and *var1::COX3* chimeric genes would exhibit post-translational defects.

Pulse-labeling of cells containing chimeric genes indicated that the rate of Cox2p synthesis directed by the *var1::COX2* mRNA was equivalent to that directed by the wild-type *COX2* mRNA. Thus, the *VAR1* 5′-UTL and 3′-UTR were fully competent in translation of this membrane protein. In sharp contrast to the normal rate of Cox2p synthesis in cells containing the *var1::COX2* chimeric mRNA, we found that those cells had severely reduced rates of respiration and growth on nonfermentable carbon sources. Furthermore, cytochrome *c* oxidase activity and the steady-state level of Cox2p were also roughly an order of magnitude lower than wild-type. Taken together, these data can be most easily explained by a model (Figure 5) in which the *var1::COX2* chimeric mRNA is translated at a location, perhaps in the matrix, that makes it difficult for Cox2p to assemble efficiently into cytochrome *c* oxidase complexes in the inner membrane. The unassembled Cox2p must be degraded rapidly, a phenomenon that has been observed previously (Dowhan *et al*., 1985). Apparently, translation of the Cox2p coding sequence under the direction of untranslated regions from an mRNA that normally encodes a soluble protein, causes mislocalized translation leading to a physiological defect. We conclude that untranslated sequences of the wild-type *COX2* mRNA are important for targeting Cox2p for correct insertion in the inner membrane, presumably through the interaction of Pet111p with the *COX2* mRNA 5′-UTL.

Although our results show that productive synthesis of Cox2p cannot be supported by the 5′- and 3′-UTRs of the *VAR1* mRNA, the apparently correct processing of newly synthesized pre-Cox2p in the *var1::COX2* strain (Figure 2) suggests that translation from the chimeric mRNA does not prevent translocation through the inner membrane. If the assumption is correct that translation of the chimeric mRNAs with *VAR1* leaders initiates on free ribosomes, then the hydrophobic, membrane-spanning domains of nascent Cox2p are apparently sufficient to cause insertion into the phospholipid bilayer of the inner membrane. This insertion, while allowing processing of the pre-Cox2p leader peptide, could either be aberrant or occur in the wrong place, thus preventing efficient assembly.

The apparent rate of Cox3p synthesis directed by the *var1::COX3* chimeric mRNA was ~60–70% lower than that directed by the wild-type *COX3* mRNA, but was nevertheless robust. The reason for the reduced pulse-labeling of Cox3p in the *var1::COX3* strain remains unclear, but it may reflect instability of the protein product. In any event, the steady-state level of Cox3p was reduced far more, relative to wild-type, than the rate of Cox3p synthesis. Thus, translation of the *var1::COX3* mRNA also appears to yield an unstable product due to mislocalized translation (Figure 5), suggesting that membrane targeting of Cox3p is normally strongly dependent on the interaction of the Pet54p–Pet122p–Pet494p complex with the *COX3* mRNA 5′-UTL.

These results demonstrate that the UTRs of the *COX2* and *COX3* mRNAs contain information necessary for normal membrane localization and/or assembly of their protein products. However, it is important to emphasize that translation of a mitochondrial mRNA under the control of membrane-bound activators is not sufficient to cause an otherwise soluble protein to insert into the inner membrane. The synthetic mitochondrial coding sequence $ARG8^{m}$ specifies the same soluble matrix protein as the wild-type nuclear gene *ARG8* (Steele *et al*., 1996). When this sequence was translated from a chimeric mRNA with the UTRs of *COX3* mRNA, the resulting $Arg8^{m}p$ was soluble (Steele *et al*., 1996). When a derivative of this sequence, which lacks the first 21 codons specifying the matrix targeting signal, was translated from a mitochondrial chimeric mRNA with the *COX2* mRNA UTRs, most

of the product was soluble and none of it was inserted into the membrane (He and Fox, 1997). Distinct signals for the translocation of Cox2p N- and C-termini through the inner membrane are indeed present within the Cox2p precursor protein (He and Fox, 1997).

Our results suggest that the *VAR1* mRNA 5′-UTL is recognized either by a soluble translational activator, or directly by free mitochondrial ribosomes. Genetic studies of *VAR1* mRNA translation have previously been hampered by the fact that Var1p is required globally for mitochondrial translation. However, it is now possible to screens for mutations that affect the expression of the *var1::ARG8^m* reporter gene in a strain expressing *VAR1^u* from the nucleus. Such studies should reveal *VAR1* mRNA-specific translational activators if they exist.

Genetic evidence for mRNA-specific translational activation mediated through 5′-UTLs has also been obtained for chloroplasts in both *Chlamydomonas* and maize (Gillham *et al*., 1994; Rochaix, 1996; Cohen and Mayfield, 1997). In addition, several RNA-binding proteins which could play a role in this process have been detected biochemically, and one of them is associated with a chloroplast membrane fraction (Zerges and Rochaix, 1998). Thus, the chloroplast genetic system may employ a mechanism for targeting membrane protein translation similar to that of *S.cerevisiae* mitochondria.

Protein localization mediated by signals in mRNAs is certainly not restricted to eukaryotic organellar systems. mRNA localization appears to be important in targeting cytoplasmically translated proteins in eukaryotic cells (Deshler *et al*., 1997; Lithgow *et al*., 1997; Chicurel *et al*., 1998). Furthermore, it was recently reported that the Type III secretion machinery of the Gram-negative bacterial pathogen *Yersinia enterocolitica* recognizes signals embedded in the RNA sequence of the first 15 codons of the mRNAs for the secreted *Yersinia* outer membrane proteins YopE and YopN (Anderson and Schneewind, 1997). These signals, present in the translated portion of the mRNAs, are sufficient to signal secretion of downstream polypeptide and are also necessary for efficient translation of the mRNAs. Whereas there is no direct evidence for a homologous relationship between bacterial Type III secretion systems and organellar translational activation systems, the similarities between them are intriguing.

Materials and methods

Yeast strains, growth conditions, DNA manipulations and the VAR1^u plasmid

The yeast strains used in this study are listed in Table I. Complete glucose (YPD), galactose (YPGal) and glycerol–ethanol (YPGE) were prepared using 2% glucose, 2% galactose or 2% glycerol plus 2% ethanol, respectively (Sherman *et al*., 1986). Minimal medium (0.67% yeast nitrogen base without amino acids) was supplemented with specific amino acids, uracil and adenine as required, and a carbon sources as specified for rich medium. Standard genetic methods (Sherman *et al*., 1986; Fox *et al*., 1991) were used. Transformation of yeast cells with nuclear plasmids was performed using the lithium acetate method (Ito *et al*., 1983) or a modification (Elble, 1992). The *VAR1^u* yeast expression vector, pEVA1, was constructed by ligation of a 3.1-kb *Sal*I fragment from pAM2 (Sanchirico *et al*., 1995) into *Sal*I digested TVS30A. pEVA1 carries the *VAR1^u* open reading frame (ORF) fused to the *COX4* mitochondrial targeting sequence under the transcriptional control of the *ADH1* promoter (UAS_{ADH1}), 2m replication origin, *LEU2* and *ADE3*. Standard techniques were used for all DNA manipulations and *Escherichia coli* transformations (Sambrook *et al*., 1989). Restriction enzymes and Vent DNA polymerase were used as recommended by the supplier (New England Biolabs, Beverly, MA). Plasmid DNA was isolated using Qiagen columns, and DNA fragments were isolated from 0.8% agarose gels using the Quiax II extraction kit (Qiagen, Chatsworth, CA). PCR amplification was performed using standard PCR conditions in either a PTC-100 (MJ Research, Watertown, MA) or Progene thermal cycler (Techne, Princeton, NJ). DNA was sequenced with the Sequenase, version 2.0 kit (United States Biomedical Corp., Cleveland, OH). The nucleotide sequence of the synthetic *VAR1^{u}ORF* is available in the EMBL Nucleotide Sequence Database (accession No. AJ010480).

Construction of var1::ARG8^m, var1::COX2 and var1::COX3 chimeric genes

Purified mtDNA was used as a template for PCR amplification of sequences from the *VAR1* locus. For the *var1::ARG8^m* construct, the downstream *VAR1* sequence containing the last 166 bp of the ORF including the stop codon was generated by PCR using primers that added a *Bam*HI site to the 5′ end and a *Pst*I site at the 3′ end of PCR amplification product. The *Bam*HI and *Pst*I sites were used to insert the fragment into *Bam*HI–*Pst*I-digested pUC19, creating pMES5. The sequence from 8 bp upstream of the $tRNA^{Ser}$ to +60 of the *VAR1* ORF was generated by PCR with primers that added an *Eco*RI site to the upstream 5′ end and *Acc*I and *Bam*HI sites to the downstream 3′ end of the fragment. This DNA fragment was digested with *Eco*RI and *Bam*HI and ligated into *Eco*RI–*Bam*HI-digested pMES5 to create pMES6. The 1.3 kb *Acc*I–*Bam*HI fragment from pDS24 containing the sequence for the *ARG8^m* gene (Steele *et al*., 1996) was then ligated to *Acc*I–*Bam*HI-digested pMES6 to create the *var1::ARG8^m* gene fusion plasmid, pMES7. This construct encodes for a fusion protein that contains the first 20 amino acids from Var1p followed by an in-frame fusion of the precursor $Arg8^m$p.

To construct the *var1::COX2* locus, a DNA fragment extending from –434 to –3 with respect to the ATG of *VAR1 ORF* and a second DNA fragment containing the last 166 bp of the *VAR1* ORF including the *VAR1* stop codon were generated by PCR amplification using two sets of primers. The primers were designed so that 18 bp at the 3′ end of the upstream fragment would overlap 18 bp at the 5′ end of the downstream fragment. The region of overlap contained *Nco*I and *Xba*I restriction sites. These two PCR-amplified DNA fragments were used as templates in a second PCR amplification using the two primers that hybridize to the 5′ end of the –434 to –3 fragment and to the 3′ end of the *VAR1* ORF fragment. The sequence overlap between the two fragments made it possible to obtain a 661 bp DNA fragment containing the mtDNA region upstream of the *VAR1* ORF, the *Nco*I and *Xba*I restriction sites, and the last 166-bp of the *VAR1* ORF. This 661 bp fragment was amplified so that *Eco*RI and *Pst*I restriction sites introduced at the ends of the fragment could be used to ligate the DNA into *Eco*RI–*Pst*I-digested pUC19 to create pMES17. The complete *COX2* ORF from the ATG through the stop codon was amplified by PCR from the plasmid pJM2 (Mulero and Fox, 1994) using primers that introduced an *Afl*III site at the ATG and an *Xba*I site directly downstream of the stop codon. The amplified *COX2* DNA fragment was digested with *Afl*III and *Xba*I and ligated into *Nco*I–*Xba*I-digested pMES17 to create the *var1::COX2* gene fusion plasmid pMES18. The 5′-end fusion between the *VAR1* UTL and the *COX2* ORF was exactly at the ATG with the ATG context altered from TAATG to CCATG.

To construct the *var1::COX3* locus, the sequence from 8-bp upstream of the $tRNA^{Ser}$ to +3 of the *VAR1* ATG was generated by PCR with primers that added an *Eco*RI site to the upstream 5′ end and the sequence for codons 2–5 of *COX3* and *Bgl*II and *Xba*I sites to the downstream 3′ end of the fragment. This DNA fragment was digested with *Eco*RI and *Xba*I and ligated into *Eco*RI–*Xba*I-digested pUC19 to create pMES19. The sequence containing the last 166 bp of the *VAR1* ORF including the stop codon was generated by PCR using primers that added *Bgl*II and *Xba*I sites to the 5′ end and a *Pst*I site at the 3′ end of PCR amplification product. The *Bgl*II and *Pst*I sites were used to insert the fragment into *Bgl*II–*Pst*I-digested pMES19 creating pMES20. pMES20 contains the sequence from 8 bp upstream of $tRNA^{Ser}$ through the *VAR1* ATG and followed downstream by in-frame codons 2–5 from *COX3*, the *Bgl*II and *Xba*I sites, and the last 166 bp of the *VAR1* ORF. The sequence from bp 22 in the *COX3* ORF downstream to the stop codon was PCR-amplified from pLSF600 (Folley and Fox, 1991) using primers that added *Bgl*II and *Xba*I sites to the upstream and downstream ends, respectively. This *COX3* DNA fragment was digested with *Bgl*II and *Xba*I and ligated into *Bgl*II–*Xba*I-digested pMES20 to create the *var1::COX3* gene fusion plasmid pMES21. The *Bgl*II site in *COX3* introduced

from the PCR primer sequence replaced bp 16–21 in the *COX3* ORF and changed the codon at position seven from AGT(Ser) to TCT(Ser).

It is important to note that translation of Cox2p and Cox3p from the chimeric mRNAs begins at the normal intiation codons and terminates at the normal termination codons present in the *COX2* and *COX3* coding sequences, respectively. Moreover, the 166 bp segment of the *VAR1*-coding sequence in the chimeric genes is out of frame with respect to the upstream *COX* coding sequences.

Mitochondrial transformation and gene replacement

High-velocity microprojectile bombardment cotransformation was performed as described (Fox *et al*., 1988b) with a helium-charged Biolistics PDS-1000 (Dupont, Boston, MA). The recipient strain was DFS160 rho^0 and the nuclear selectable plasmid was YEp351 (Hill *et al*., 1986). For *var1::ARG8*^{m}, pMES7 was cotransformed and Leu$^+$ nuclear transformants were replica mated to a lawn of DFS188 containing the *VAR1*^{u} plasmid pEVA1. The mated cells were then replica plated to SGE lacking Arg to identify mitochondrial transformants carrying pMES7 and to select for diploid mitochondrial recombinants in which *var1::ARG8*^{m} was integrated at the *VAR1* locus in mtDNA. The stable synthetic rho^- strains were purified through five to ten rounds of subcloning. The replacement of *VAR1* by the *var1::ARG8*^{m} construct was confirmed by Southern analysis, and as expected, mitochondrial translation in the *var1::ARG8*^{m} strain was dependent on the presence of the *VAR1*^{u} expression plasmid pEVA1.

For *var1::COX2* and *var1::COX3*, DFS160 rho^0 was cotransformed as above with pMES18 or pMES21, respectively. Leu$^+$ nuclear transformants were replica mated to lawns of testers strains TF192 or LSF5, and the mated cells were then replica plated to YPGE to detect marker rescue by synthetic rho^- mitochondrial transformants carrying either pMES18 or pMES21, respectively. Stable synthetic rho^- strains were purified through three to five rounds of subcloning. To integrate the *var1::COX2* and the *var1::COX3* constructs into rho^+ mtDNA, the synthetic rho^- strains MSY493 and MSY523 were mated, respectively, to the respiratory deficient strains HMD22 [*arg8* (*rho*$^+$ *cox2::ARG8*^{m})] and DFS189 [*arg8* (*rho*$^+$ *cox3::ARG8*^{m})], each of which carried the *VAR1*^{u} expression plasmid pEVA1. Diploids with either the *var1::COX2* or the *var1::COX3* allele integrated at the *VAR1* locus were identified by their ability to grow, albeit slowly, on YPGE. Each was confirmed by Southern analysis and dependence of mitochondrial translation on the presence of the *VAR1*^{u} expression plasmid pEVA1.

Isolation of mitochondria, mtDNA and immunological analysis

Yeast cells were grown to mid-exponential phase in YPGal medium and mitochondria were isolated as described by Daum *et al*. (1982). Mitochondrial DNA was isolated by resuspending purified mitochondria in lysis buffer (50 mM Tris–HCl pH 7.5, 300 mM NaOAc pH 5.2, 10 mM EDTA, 2% SDS), extracting three times with phenol, twice with phenol–chloroform, and once with chloroform. Mitochondrial DNA was precipitated from the aqueous phase with ethanol. Immunoblots were performed using the culture supernatants from the CCO6 and CCO20 hybridoma cell lines that secrete antibodies to Cox2p and Cox3p, respectively. Immunoblot analysis was performed as described previously (Fearon and Mason, 1988). ^{125}I-labeled goat anti-mouse IgG (Amersham, Arlington Heights, IL) was used to decorate immune complexes.

Labeling of mitochondrial translation products

In vivo pulse-labeling of mitochondrial translation products with [^{35}S]-methionine Trans label (ICN Biomedicals, Irvine, CA) was essentially as described previously (Fox *et al*., 1991), except that log-phase cells were incubated in the presence of 1 mCi Translabel for 5 or 10 min at 30°C in the presence of 500 μg per ml cycloheximide. Labeling was stopped by rapid chilling of the cells by dilution into 12 ml of crushed ice and water and all subsequent steps were performed on ice. Mitochondria were isolated from labeled cells after breakage by vortexing with glass beads as described by Douglas *et al*. (1979). The radiolabeled proteins were analyzed by SDS–PAGE in a 12.5% gel and analysis of the dried gel using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantitation of the radioactivity was performed using the ImageQuant v1.1 program with local average background correction (Molecular Dynamics, Sunnyvale, CA).

Assays of whole-cell respiration and cytochrome c oxidase

Cyanide-sensitive respiration of whole cells was measured polarographically with a Clark electrode essentially as described by Rouslin and Schatz (1969). Cells were grown to mid-exponential phase in YPGal liquid medium at 30°C. Cells in ~5 ml culture medium were harvested by centrifugation, resuspended in 500–750 ml of respiration buffer (40 mM KPO_4 pH 7.4, 0.2% glucose, 1% ethanol) and stored on ice. Measurements were made with 50–200 ml of the cell suspension injected into the respiration chamber containing 1.5 ml of air-saturated respiration buffer at 30°C. Cyanide-sensitive oxygen uptake was measured in the presence of 1.0 mM KCN. The cell concentration was estimated by optical density at 600 nm. The rate of cyanide-sensitive respiration was expressed as the change in the percent O_2 saturation per minute per OD_{600}. Protein concentrations were determined according to Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard. Cytochrome *c* oxidase activity was determined spectrophotometrically as described previously (Mason *et al*., 1973).

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