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Aberrant Translation of Cytochrome *c* Oxidase Subunit 1 mRNA Species in the Absence of Mss51p in the Yeast *Saccharomyces cerevisiae*

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Expression of yeast mitochondrial genes depends on specific translational activators acting on the 5′-untranslated region of their target mRNAs. Mss51p is a translational factor for cytochrome *c* oxidase subunit 1 (*COX1*) mRNA and a key player in down-regulating Cox1p expression when subunits with which it normally interacts are not available. Mss51p probably acts on the 5′-untranslated region of *COX1* mRNA to initiate translation and on the coding sequence itself to facilitate elongation. Mss51p binds newly synthesized Cox1p, an interaction that could be necessary for translation. To gain insight into the different roles of Mss51p on Cox1p biogenesis, we have analyzed the properties of a new mitochondrial protein, mp15, which is synthesized in *mss51* mutants and in cytochrome oxidase mutants in which Cox1p translation is suppressed. The mp15 polypeptide is not detected in *cox14* mutants that express Cox1p normally. We show that mp15 is a truncated translation product of *COX1* mRNA whose synthesis requires the *COX1* mRNA-specific translational activator Pet309p. These results support a key role for Mss51p in translationally regulating Cox1p synthesis by the status of cytochrome oxidase assembly.

INTRODUCTION

Biogenesis of eukaryotic cytochrome *c* oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, involves the coordinated action of two genomes. Three proteins, encoded in the mitochondrial DNA (mtDNA), form the catalytic core with the heme A and copper prosthetic groups of the enzyme. The other nine to 10 subunits, encoded in nuclear DNA, interact with the core subunits to form a stable COX complex. In addition to the structural subunits, COX biogenesis requires the assistance of at least 20 other nuclear gene products some of which are essential for the expression of the mitochondrial encoded subunits (McEwen *et al*., 1986; Tzagoloff and Dieckmann, 1990; Barrientos *et al*., 2002a; Solans *et al*., 2004).

In *Saccharomyces cerevisiae*, translation of each mitochondrial COX mRNA depends on one or more translational activator (for review, see Towpik, 2005). These mRNA-specific translational factors are either integral or peripheral inner membrane proteins that recognize the 5′-untranslated region (UTR) of their target transcripts. Current speculations suggest that translational activators may couple translation to cotranslational insertion of the newly synthesized hydrophobic products into the membrane near or at the site of their assembly into multisubunit complexes (Michaelis *et al*., 1991; Naithani *et al*., 2003). Most activators, Mss51p being an exception, interact with each other (Brown *et al*., 1994; Naithani *et al*., 2003), suggesting some level of coregulation in the expression of the core-forming subunits of COX (Fiori *et al*., 2005). Interactions have also been noted between the transcription factor Nam1p and translational activators, including Pet111p, Pet309p, and Pet494p (Naithani *et al*., 2003; Krause *et al*., 2004) raising the possibility that mitochondrial transcription may be coupled to translation.

COX1 is the mitochondrial gene for subunit1/Cox1p, an important catalytic subunit of COX containing copper and heme A prosthetic groups. Translation of *COX1* in *S. cerevisiae* is under the control of the *MSS51* and *PET309* gene products that are also involved in maturation of the *COX1* mRNAs (Decoster *et al*., 1990; Manthey and McEwen, 1995). Mutations in *MSS51* or overexpression of the wild type gene can suppress *shy1* null mutants by enhancing synthesis of Cox1p (Barrientos *et al*., 2002b). The function of Shy1p in maturation and/or assembly of Cox1p (Barrientos *et al*., 2002b; Perez-Martinez *et al*., 2003; Smith *et al*., 2005) is of considerable interest because mutations in its human homologue, Surf1p, are responsible for most diagnosed cases of Leigh's syndrome presenting a COX deficiency (Tiranti *et al*., 1998; Zhu *et al*., 1998).

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Abbreviations used: CAP, chloramphenicol; COX, cytochrome *c* oxidase; GST, glutathione *S*-transferase; UTR, untranslated region; Y3H, yeast three-hybrid system.

Cox1p synthesis is suppressed in most COX assembly mutants, including *shy1* mutants, but it is restored to normal levels by *mss51* suppressors of *shy1* or by mutations in *COX14* (Barrientos *et al*., 2004), which codes for a COX assembly factor (Glerum *et al*., 1995). Like other translational activators Mss51p acts on the 5′-UTR to initiate translation (Perez-Martinez *et al*., 2003); however, *mss51* mutants, unlike *pet309* mutants, cannot be suppressed by changes in the 5′-UTR of *COX1* mRNA. (Perez-Martinez *et al*., 2003; Barrientos *et al*., 2004). In addition, Mss51p acts on a target in the protein coding sequence of *COX1* mRNA to promote elongation (Perez-Martinez *et al*., 2003). Mss51p and Cox1p form a transient complex (Perez-Martinez *et al*., 2003; Barrientos *et al*., 2004) that is stabilized by Cox14p (Barrientos *et al*., 2004). These interactions have been postulated to down-regulate Cox1p synthesis when COX assembly is impaired (Barrientos *et al*., 2004). According to this model, the release of Mss51p from the ternary complex and its availability for Cox1p synthesis occur at a downstream step in the assembly pathway, most likely catalyzed by Shy1p (Barrientos *et al*., 2004). Further studies are necessary to clarify the functional significance of the interactions of Mss51p on the 5′-UTR and the coding sequence of *COX1* mRNA.

In the present study, we report a novel mitochondrial translation product, named mp15, with an apparent mass of ~15 kDa. This protein is detected in *mss51* null mutants blocked in Cox1p synthesis and in strains carrying null alleles in nuclear genes coding for COX subunits or assembly factors in which the synthesis of Cox1p is reduced to 10% or less of wild type. We present evidence that mp15 is a truncated translation product of a partially unprocessed *COX1* mRNA and that it is synthesized by an Mss51p- but not Pet309-independent mechanism. We also present evidence that binding of Mss51p to the 5′-UTR of *COX1* mRNA is necessary for optimal initiation of translation by Pet309p, whereas the interaction of Mss51p with newly synthesized Cox1p may regulate elongation of the nascent polypeptide.

MATERIALS AND METHODS

Strains and Media

The genotypes and sources of the *S. cerevisiae* strains carrying null alleles of COX-related genes are listed in Table 1. Double mutants were constructed by crosses of the single mutants. The compositions of the growth media have been described previously (Myers *et al*., 1985).

In Vivo Mitochondrial Protein Synthesis

Mitochondrial gene products were labeled with [^{35}S]methionine (7 mCi/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) in whole cells at 30°C in the presence of cycloheximide (Barrientos *et al*., 2002b). Equivalent amounts of total cellular proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to a Kodak X-OMAT x-ray film. Deviations from this procedure are described in some figure legends.

Construction of COX Mutant Strains Carrying a Proteolytically Inactive Version of yta10

The haploid strain W303Δ*yta10/Yta10*^{E559Q} (Table 1) was obtained from Prof. T. Langer (Institut für Genetik und Zentrum für Molekulare Medizin, Universität zu Köln, Köln, Germany). This strain was crossed to a W303Δ*shy1* strain and to W303Δ*cox11*. Diploid cells were sporulated and haploid progeny with the two parental mutant alleles (*yta10* + *shy1* or *yta10* + *cox11*) and the *yta*^{10E559Q} allele were isolated.

Protein Purification and Sequencing

Mss51p fused with the 26-kDa glutathione S-transferase (GST) was expressed from an integrative plasmid (pG96/ST13) in a strain carrying a null mutant allele of *mss51* as reported previously (aW303Δ*mss51*/ST13) (Barrientos *et al*., 2004). This strain was respiratory competent and grew on nonfermentable carbon sources with a doubling time similar to the parental wild type strain (Barrientos *et al*., 2004). Mitochondria were prepared from aW303Δ*mss51*/ST13 strain by the method of Faye *et al*. (1974) except that zymolyase 20,000 (ICN Biochemicals, Aurora, OH) instead of glusulase was used to digest the cell wall. Mitochondrial proteins (5 mg) were solubilized with 1% lauryl maltoside in the presence of 0.5 M KCl and 1 mM phenylmethylsulfonyl fluoride. The extract was clarified by centrifugation at 50,000 × g_{av} for 30 min and incubated with glutathione-Sepharose beads for 4 h at 4°C. After centrifugation at 1500 rpm for 5 min, the beads were washed three times with phosphate-buffered saline. The Mss51p–GST fusion protein was eluted with 10 mM glutathione, 50 mM Tris-base, pH 8.0, and concentrated using Vivaspin 500 columns. The concentrate was separated in a 12% SDS-PAGE system, transferred to a polyvinylidene fluoride membrane and stained with Coomassie blue. A prominent band of ~68 kDa was excised from the membrane and used to sequence the N-terminal seven residues with a Procise Sequencer (Applied Biosystems, Foster City, CA) in the Proteomics facility of the University of Miami.

Yeast Three-Hybrid System (Y3H)

A Y3H system to map the interacting domain in Mss51p and its target in the 5′-UTR of *COX1* mRNA was developed by using the commercially available RNA-Protein Hybrid Hunter kit (Invitrogen, Carlsbad, CA). Portions of the *COX1* mRNA 5′-UTR were cloned into the plasmid pRH3′ and portions, of Mss51p were expressed from the plasmid pYESTrp2. The host strain L40-ura-ms2 was cotransformed with pairs of pRH3′/and pYESTrp2/constructs, and the transformants were selected on yeast synthetic medium missing the appropriate auxotrophic markers (Ura− and Trp−) and histidine. The His−-deficient medium selected for expression of the *HIS3* reporter gene, e.g., for colonies in which the RNA–protein interaction was produced. Inclusion of 5 mM 3-minotriazole, a competitive inhibitor of the *HIS3* reporter protein, suppressed background growth in the His− medium. Cotransformations of the host strain with pair-combinations of the parent plasmids and one of the constructs served as controls. Interactions were confirmed by assaying for β-galactoside, the second reporter using a filter lift assay as described previously (Breeden and Nasmyth, 1985).

Miscellaneous Procedures

Standard procedures were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *Escherichia coli* (Sambrook *et al*., 1989). Yeast were transformed by the method of Schiestl and Gietz (1989). The one-step gene insertion method (Rothstein, 1983) was used to integrate linear plasmids at the *URA3* or *LEU2* locus of yeast nuclear DNA. Mitochondrial RNA was prepared from isolated mitochondria by modified extraction with hot-acidic phenol (Ausubel *et al*., 1994).

RESULTS

Synthesis of a Novel Polypeptide from a COX1 Transcript in mss51 Null Mutants

PET309 and *MSS51* code for inner membrane proteins that are essential for translation of mitochondrial *COX1* mRNA in *S. cerevisiae* (Decoster *et al*., 1990; Manthey and McEwen, 1995). In agreement with earlier studies, mutations in either of these genes abolish synthesis of Cox1p (Figure 1*A*). However, a new low-molecular-weight polypeptide with a migration intermediate between Atp6p and Atp8p in SDS-PAGE was detected among the mitochondrial translation products labeled with [^{35}S]methionine in the *mss51* null mutant. Expression of wild-type *MSS51* from integrative and episomal plasmids in this *mss51* null mutant strain completely restored its ability of synthesizing Cox1p and abolished the synthesis of the new polypeptide (data not shown). This protein was absent in the *pet309* mutant and in a *pet309/mss51* double mutant (Figure 1A). The absence of the novel polypeptide in the *pet309* mutant suggested that it could be a truncated product of *COX1* requiring Pet309p for initiation of translation but destined for premature termination in the absence of Mss51p. To further establish the origin of the new polypeptide, mtDNA of M5-16/A3, a mutant with a large deletion in *COX1* (Tzagoloff *et al*., 1975) was transferred by cytoduction to an *mss51* null mutant and its parental wild type, both lacking mtDNA (ρ^o strains). No synthesis of Cox1p or the novel protein was seen in the *cox1* mutant either in the presence or absence of the *mss51* null mutation (Figure 1B). These data constitute strong evidence

Table 1. Genotypes and sources of yeast strains

Strain	Strains with mitochondrial DNA of different source and intron composition: Genotype	mtDNA	Source
W303-1A	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI5γ^+	R. Rothstein (Department of Human Genetics, Columbia University, New York, NY)
W303-1B	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI5γ^+	R. Rothstein
W303/ρ0	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^o	This study
D273-10B/A1	**MAT**a *met6*	ρ^+ bI+, aI1+, aI2+, aI3+, aI4+, aI5g$^+$	Tzagoloff *et al.* (1976)
JC3/ρ^o	**MAT**a *kar1-1 ade2 lys2*	ρ^o	ATCC 201577
JC3/ρ^{D273}	**MAT**a *kar1-1 ade2 lys2*	ρ^+ bI+, aI1+, aI2+, aI3+, aI4+, aI5g$^+$	JC3 ρ^o × D273-10B/A21
JC11/r^{CK5112}	**MAT**α, *kar1-1, his3*	ρ^+ aI2$^+$, aI3$^+$, aI5γ^+ *w*$^+$	J. Lazowska (Centre de Génétique Moleculaire du Centre National de la Recherche Scientifique, Gif-sur-Yvette, France)
JC11/r^{WI04}	**MAT**α, *kar1-1, his3*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI5 $^+$	Labouesse (1990)
JC3/r$^{GF134-6D}$	**MAT**a *kar1-1 ade2 lys2*	ρ^+ bI$^+$, aI4$^+$	Barros *et al.* (2006)
D273-10B/ρ^{G1-224}	**MAT**α *met6*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI4$^+$	Seraphin *et al.* (1987)
JC3/ρ^{G1-224}	**MAT**a *kar1-1 ade2 lys2*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI4$^+$	JC3ρ^o × D273-10B/G1-224
D273-10B/$\rho^{G1-356-R5}$	**MAT**α *met6 lys2*	ρ^+ bI$^+$, aI1$^+$, aI4$^+$, aI5g$^+$	Seraphin *et al.* (1987)
JC3/$\rho^{G1-356-R5}$	**MAT**a *kar1-1 ade2 lys2*	ρ^+ bI$^+$, aI1$^+$, aI4$^+$, aI5g$^+$	JC3ρ^o × D273-10B/G1-356-R5
aW303/ρ^{Io}	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ I^o	Barros *et al.* (2005)
JC3/ρ^{W303}	**MAT**a *kar1-1 ade2 lys2*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI5γ^+	JC3ρ^o × W303-1B
Δ*mss51*/ρ^{D273}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ bI+, aI1+, aI2+, aI3+, aI4+, aI5g$^+$	W303Δ*mss51* × JC3/ρ^{D273}
Δ*mss51*/ρ^{G1-224}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI4$^+$	W303Δ*mss51* × JC3/ρ^{G1-224}
Δ*mss51*/$\rho^{G1-356-R5}$	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ bI$^+$, aI1$^+$, aI4$^+$, aI5g$^+$	W303Δ*mss51* × JC3/$\rho^{G1-356-R5}$
Δ*mss51*/ρ^{CK5112}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ aI2$^+$, aI3$^+$, aI5γ^+ *w*$^+$	W303Δ*mss51* × JC3/ρ^{CK5112}
Δ*mss51*/$\rho^{GF134-6D}$	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ bI$^+$, aI4$^+$	W303Δ*mss51* × JC3/$\rho^{GF134-6D}$
Δ*mss51*/ρ^{WI04}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI5γ^+	W303Δ*mss51* × JC11/ρ^{WI04}
Δ*mss51*/ρ^{Io}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*mss51::HIS3*	ρ^+ I^o	W303Δ*mss51* × W303/ρ^{Io}
Δ*shy1*/ρ^{G1-224}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*shy1::URA3*	ρ^+ bI$^+$, aI1$^+$, aI2$^+$, aI3$^+$, aI4$^+$	W303Δ*mss51* × JC3/ρ^{G1-224}
Δ*shy1*/$\rho^{G1-356-R5}$	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*shy1::URA3*	ρ^+ bI$^+$, aI1$^+$, aI4$^+$, aI5g$^+$	W303Δ*mss51* × JC3/$\rho^{G1-356-R5}$
Δ*shy1*/ρ^{CK5112}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*shy1::URA3*	ρ^+ Ai2$^+$, aI3$^+$, aI5γ^+ *w*$^+$	W303Δ*mss51* × JC3/ρ^{CK5112}
Δ*shy1*/$\rho^{GF134-6D}$	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*shy1::URA3*	ρ^+ Bi$^+$, aI4$^+$	W303Δ *mss51* × JC3/$\rho^{GF134-6D}$
Δ*shy1*/ρ^{Io}	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*shy1::URA3*	ρ^+ I^o	W303Δ *mss51* × JC3/ρ^{Io}
Structural subunits[b]			
W303Δ*cox4*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*cox4::URA3*	W303	Glerum and Tzagoloff (1997)
W303Δ*cox5a*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*cox5a::HIS3*	W303	Glerum and Tzagoloff (1997)
W303Δ*cox6*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*cox6::URA3*	W303	Glerum and Tzagoloff (1997)
W303Δ*cox7*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*cox7::URA3*	W303	Barrientos *et al.* (2004)
W303Δ*cox9*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* Δ*cox9::URA3*	W303	Barrientos *et al.* (2004)
M5-16-A3	*ade1 cox1*	D273	Tzagoloff *et al.* (1975)

Continued

Table 1. *Continued*

Strain[a]	Genotype	mtDNA	Source
	Strains with mutant alleles of cox assembly factors		
COX1 expression[b]			
W303Δ*pet309*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet309::HIS3*	W303	Glerum and Tzagoloff (1997)
W303Δ*mss51*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δmss51::HIS3*	W303	Barrientos *et al.* (2002b)
C199	*met6 mss51-199*	D273	Tzagoloff, unpublished data
C283	*met6 mss51-283*	D273	Tzagoloff, unpublished data
Maturation of CuA or CuB centers[b]			
W303Δ*cox17*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox17::TRP1*	W303	Glerum *et al.* (1996a)
W303Δ*sco1*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δsco1::URA3*	W303	Glerum *et al.* (1996b)
W303Δ*cox11*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox11::HIS3*	W303	Tzagoloff *et al.* (1990)
COX2 expression[b]			
W303Δ*oxa1*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δoxa1::HIS3*	W303	Hell *et al.* (2000)
W303Δ*cox18*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox18::URA3*	W303	Souza *et al.* (2000)
W303Δ*pet111*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet111::HIS3*	W303	Barros *et al.* (2002)
W303Δ*imp1*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δimp1::HIS3*	W303	Barrientos *et al.* (2004)
W303Δ*imp2*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δimp2::URA3*	W303	Barros *et al.* (2002)
Heme biosynthesis[b]			
W303Δ*cox10*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox10::HIS3*	W303	Nobrega *et al.* (1990)
W303Δ*cox15*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox15::HIS3*	W303	Glerum *et al.* (1997)
Assembly/unknown[b]			
W303Δ*pet117*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet117::HIS3*	W303	Barros *et al.* (2002)
W303Δ*shy1*/U2	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δshy1::URA3*	W303	Barrientos *et al.* (2002b)
W303Δ*pet191*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet191::HIS3*	W303	Barrientos *et al.* (2004)
W303Δ*cox14*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox14::TRP1*	W303	Barrientos *et al.* (2004)
W303Δ*cox16*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox14::URA3*	W303	Carlson *et al.* (2003)
W303Δ*cox19*	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox19::URA3*	W303	Nobrega *et al.* (2002)
	Strains carrying mutant alleles of yta10		
YHA103	*MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112* YCplac111 *(LEU2, CEN):ADH1-YTA10^{E559Q} ura3-52Δ yta10::URA3*	W303	Arlt *et al.* (1996)
W303Δ*shy1*Δ*yta10/ yta10*^{E559Q}	*MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112* YCplac111 *(LEU2, CEN):ADH1-YTA10^{E559Q} ura3-52Δ yta10Δshy1::URA3*	W303	W303Δ*shy1* × YHA103
W303Δ*cox11*Δ*yta10/ yta10*^{E559Q}	*MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112* YCplac111 *(LEU2, CEN):ADH1-YTA10^{E559Q} ura3-52Δ yta10::URA3Δ cox11::HIS3*	W303	W303Δ*cox11* × YHA103

[a] All null mutations have been created or are available in both a and α mating types.

[b] These headings indicate the functional category of the deleted gene products.

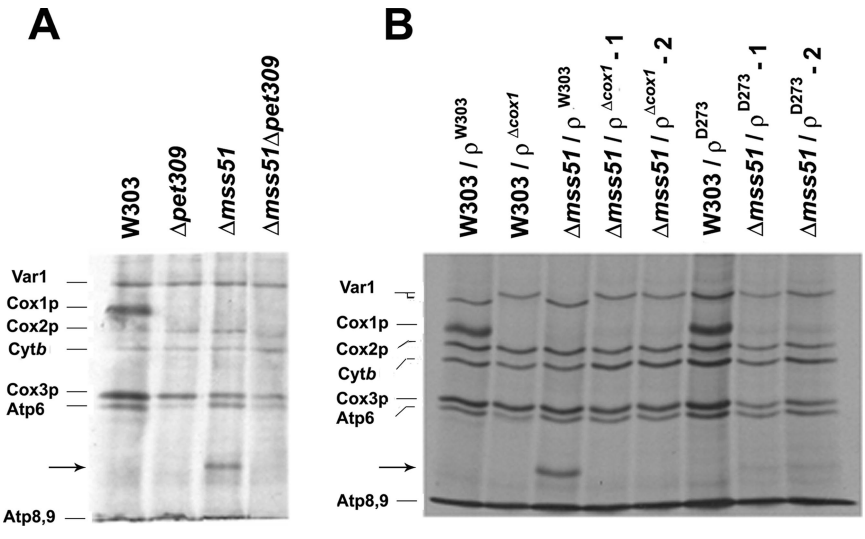

Figure 1. Pet309p and *COX1*-dependent synthesis of a novel protein in *mss51* null mutants. (A) In vivo synthesis of mp15 by *mss51* mutants requires the presence of Pet309p. Mitochondrial products of wild type (W303) and of *pet309* and *mss51* single or double mutants were labeled with [^{35}S]methionine at 30°C for 10 min in the presence of cycloheximide (Barrientos *et al.*, 2002b). (B) A partial deletion of *COX1* blocks in vivo synthesis of mp15. Mitochondrial products were labeled as in A in the wild type W303-1A (W303) and *mss51* null mutants (Δ*mss51*) in different mitochondrial genetic backgrounds: W303-mtDNA (ρ^{W303}), D273-mtDNA (W303ρ^{D273}), Δ*mss51*ρ^{D273}), or Δ*cox1*-mtDNA (Tzagoloff *et al.*, 1975), the later a D273 type of D273-mtDNA with a partial deletion of the *COX1* gene obtained from strain M5.16-A3 (Table 1). Δ*mss51*r^{Dcox1} -1 and -2 and Δ*mss51*ρ^{D273} -1 and -2 are two different cytoductants of each type.

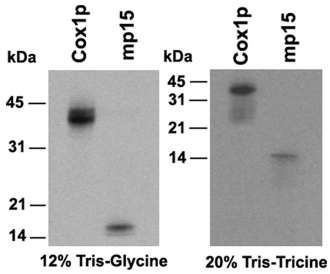

Figure 2. Electrophoretic mobility of mp15. Mitochondrial products were labeled in vivo in a wild type (W303-1A) and a mutant strain carrying a null allele of *mss51* (Δ*mss51*) as in Figure 1. Total cellular proteins were separated in a 17.5% PAGE (Barrientos *et al*., 2002b). Cox1p of the wild-type cells and mp15 from the *mss51* mutant were excised from the SDS polyacrylamide gel and electroeluted using ElutaTube Protein Extraction kit (Fermentas, Ontario, Canada). The proteins were trichloroacetic acid (TCA) precipitated and resuspended in loading buffer. The radiochemical purity of the two proteins was tested by separation on a second 12% polyacrylamide gel (Laemmli, 1970) or a 20% polyacrylamide gel in Tris-Tricine buffer (Schagger and von Jagow, 1987).

that the novel protein, which we have named mp15, is encoded by a *COX1* transcript.

The mtDNA of D273-10B, the parent of the *cox1* mutant M5-16/A3 was also transferred to the *mss51* mutant and to the isogenic wild-type strain W303-1A. The introduction of the D273 mtDNA into the W303 nuclear background of the *mss51* mutant resulted in significantly reduced synthesis and/or stability of mp15 (Figure 1B). No mp15 was detected in the parental strain with wild-type *MSS51* and D273-10B mtDNA.

The mp15 detected in *mss51* mutants could be a novel translation product of a *COX1* transcript or a degradation product of Cox1p. The fact that no Cox1p is detected in *mss51* mutants, even after pulses of up to 60 min (data not shown), suggested that there is no synthesis of full-length Cox1p. Detection of mp15 after a 2.5-min pulse, the shortest time needed to observe a clear pattern of labeled proteins in the in vivo experiments (data not shown), argues against degradation of Cox1p as the source of mp15.

Radiolabeled mp15 separated by SDS-PAGE migrates slightly above 14-kDa marker (Figure 2) indicating a molecular weight of ~15,000. Because hydrophobic proteins do not always separate true to size, the actual molecular weight could be larger.

Because mp15 is synthesized in low amounts, only detected because it is radiolabeled, and is highly labile (explained below), it is very difficult to purify enough protein to attempt mass spectrometry characterization. Attempts will be made in the future with this goal.

Pet309-dependent Synthesis of mp15 Polypeptide Occurs in COX Assembly-arrested Mutants Displaying Reduced Synthesis of Cox1p

Mss51p was proposed to play a role in adjusting translation of Cox1p to the availability of its partners subunits during assembly of COX (Barrientos *et al*., 2002b, 2004). This was based on observations that synthesis of Cox1p is greatly reduced in most COX assembly mutants (Barrientos *et al*., 2004; Figure 3, A and B). The small amount of Cox1p detected in such mutants may reflect the fraction of this subunit that is associated with and stabilized by Mss51p and Cox14p (Perez-Martinez *et al*., 2003; Barrientos *et al*., 2004). Assembly-defective mutants in which Mss51p is tied up with unassembled Cox1p would be expected to have substantially reduced amounts of Mss51p available for new rounds of Cox1p translation, thereby allowing synthesis of mp15.

In vivo labeling of mitochondrial gene products indicated that with the exception of the *cox14* mutant, all the other COX mutants tested synthesize mp15 (Figure 3A). As with the *mss51* mutant, the presence of a second mutation in *pet309* completely suppressed the synthesis of mp15 in all the COX mutants (Figure 3A). The efficiency of mp15 synthesis was variable but never as high as in the *mss51* mutant. No mp15 was detected in the *cox14* mutant, which as reported previously, synthesizes normal amounts of Cox1p (Barrientos *et al*., 2004; Figure 3, A and B). Synthesis of mp15 was also abolished or greatly reduced in double mutants in which a null allele in a COX assembly gene was combined with a deletion of *COX14* (Figure 3B). Such strains were previously shown to synthesize Cox1p normally (Barrientos *et al*., 2004).

Synthesis of mp15 in the mss51 Mutant Is Not Affected by a cox14 Mutation

Cox14p plays an important role in regulating translation of *COX1* mRNA. In other studies, we have shown that *cox14* mutants synthesize normal amounts of Cox1p. Moreover, the *cox14* mutation cures the Cox1p translational defect of most COX mutants (Barrientos *et al*., 2004). This was interpreted to indicate that Cox14p may be necessary to stabilize the interaction of Mss51p with Cox1p. Accordingly, mutants lacking Cox14p would have more Mss51p available for translation of Cox1p.

The synthesis of mp15 is independent of Cox14p, because incorporation of [^{35}S]methionine into mp15 was the same in the *mss51* single mutant and in a strain with mutation in both *mss51* and *cox14* (Figure 4A). Overexpression of *COX14* from the episomal plasmid pG93/T1 (Glerum *et al*., 1995) in a *mss51* null mutant strain also did not influence the synthesis of mp15. These results support a role of Cox14p in regulating the availability of Mss51p for translation by trapping the protein in a ternary complex with full-length Cox1p, rather than acting to inhibit translational elongation.

Accumulation of mp15 Is Significantly Reduced In Vivo in COX Assembly Mutants Pretreated with Chloramphenicol

We have previously shown that, with the exception of the *mss51*, *pet309*, and *oxa1* mutants, Cox1p labeling was increased in COX mutants when cells were preincubated in chloramphenicol (CAP) before the in vivo pulse (Barrientos *et al*., 2004), which was interpreted to be due to the accumulated larger pools of nuclear-encoded factors (i.e., Mss51p) required for mitochondrial gene expression. We wanted to test now if the enhanced synthesis of Cox1p in most COX assembly mutants had a modifying effect on the amount of newly synthesized mp15.

We first tested the effect of CAP preincubation on mp15 synthesis in *mss51* mutants. Incorporation of [^{35}S]methionine into mp15 in an *mss51* null mutant strain was substantially lower in CAP-pretreated cells (Figure 5, A and B). Although at variable levels, in some experiments, the amount of newly synthesized mitochondrial ribosomal protein Var1 was also reduced in *mss51* mutants pretreated with CAP, suggesting an effect of the antibiotic on ribosome metabolism that could be also related to the reduced synthesis of mp15. However, the amount of newly synthesized mp15 was consistently reduced in CAP-pretreated *mss51* mutant cells even in experiments in which the amount of newly synthesized Var1 was not affected. The reduction in mp15 was unlikely to be due to faster turnover, because it was observed at the shortest times of labeling. Pulse-chase experiments also showed that mp15 synthesized in 10 min (Figure 5,

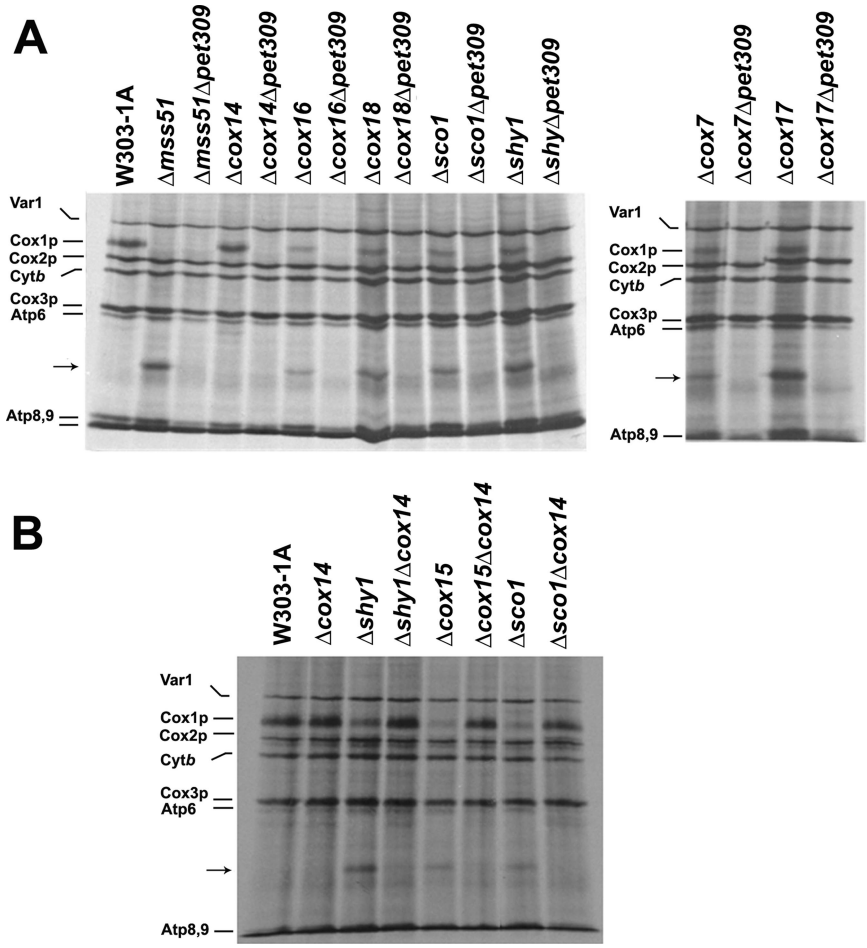

Figure 3. Accumulates of mp15 in vivo in COX assembly-arrested mutants. (A) Wild type (W303-1A), *mss51*, *cox14*, *cox16*, *cox18*, *sco1*, *cox7*, and *cox17* single null mutants or double mutants with a second null mutation in *PET309* were labeled with [^{35}S]methionine at 30°C for 15 min in the presence of cycloheximide as in Figure 1. (B) Wild type (W303-1A), *cox14* single mutant or double mutants with a second mutation in *shy1*, *cox15*, and *sco1* were labeled with [^{35}S]methionine at 30°C for 15 min in the presence of cycloheximide as in Figure 1. The mitochondrial translation products are identified in the margin. The functions affected in the different strains are described in Table 1.

B and C) or 15 min of pulse (data not shown) with or without the CAP pretreatment were degraded at similar rates.

Pretreatment with CAP of different COX assembly-defective mutants reduced incorporation of [^{35}S]methionine into mp15 to undetectable levels (Figure 5D). Synthesis of Cox1p in the CAP-treated mutants was comparable with that seen in wild type under normal pulse-labeling conditions. This confirms the presence of a fully functional translation apparatus in the COX mutants and supports the earlier conclusion that synthesis of Cox1p is down-regulated in such strains (Barrientos *et al*., 2004).

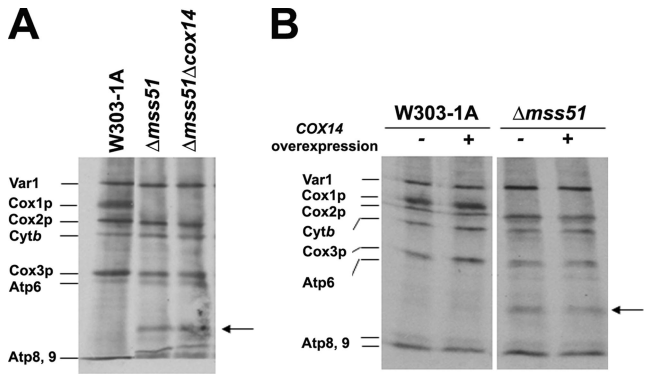

Figure 4. *COX14* does not affect synthesis and accumulation of mp15. (A) Wild type (W303-1A), an *mss51* null mutant, and an *mss5/cox14* double mutant were labeled with [^{35}S]methionine at 30°C for 15 min in the presence of cycloheximide as described in Figure 1. (B) Wild type (W303-1A), and a null mutant of *mss51* overexpressing or not *COX14*, were labeled in vivo as described in A. The mitochondrial translation products are identified in the margin.

Mp15 Is a Membrane Protein and the Yta10p/Yta12p Protease Is Partially Responsible for Its Degradation

Cox1p is an integral protein, embedded in the mitochondrial inner membrane with 12 transmembrane α-helices (Tsukihara *et al*., 1996). To test whether mp15 is also a membrane-bound protein, we have determined its solubility properties. Sonic irradiation of Δ*mss51* spheroplasts containing newly synthesized ^{35}S-labeled mitochondrial translation products solubilized α-ketoglutarate dehydrogenase, a soluble protein of the mitochondrial matrix, but not mp15 nor any of the other newly synthesized proteins that are known to be cotranslationally inserted in the membrane (Figure 6A). These results suggest that also mp15 is cotranslationally inserted in the mitochondrial inner membrane.

Our failure to detect mp15 with monoclonal antibodies against Cox1p (data not shown) suggested that either the Cox1p epitope is not present in mp15 or/and that most of this protein is degraded. Pulse-chase analysis of mp15 stability in an *mss51* null mutant indicated ~50% of the newly synthesized mp15 to be degraded after a 1-h chase (Figure 6B). The stability of unassembled Cox1p and of mp15 was

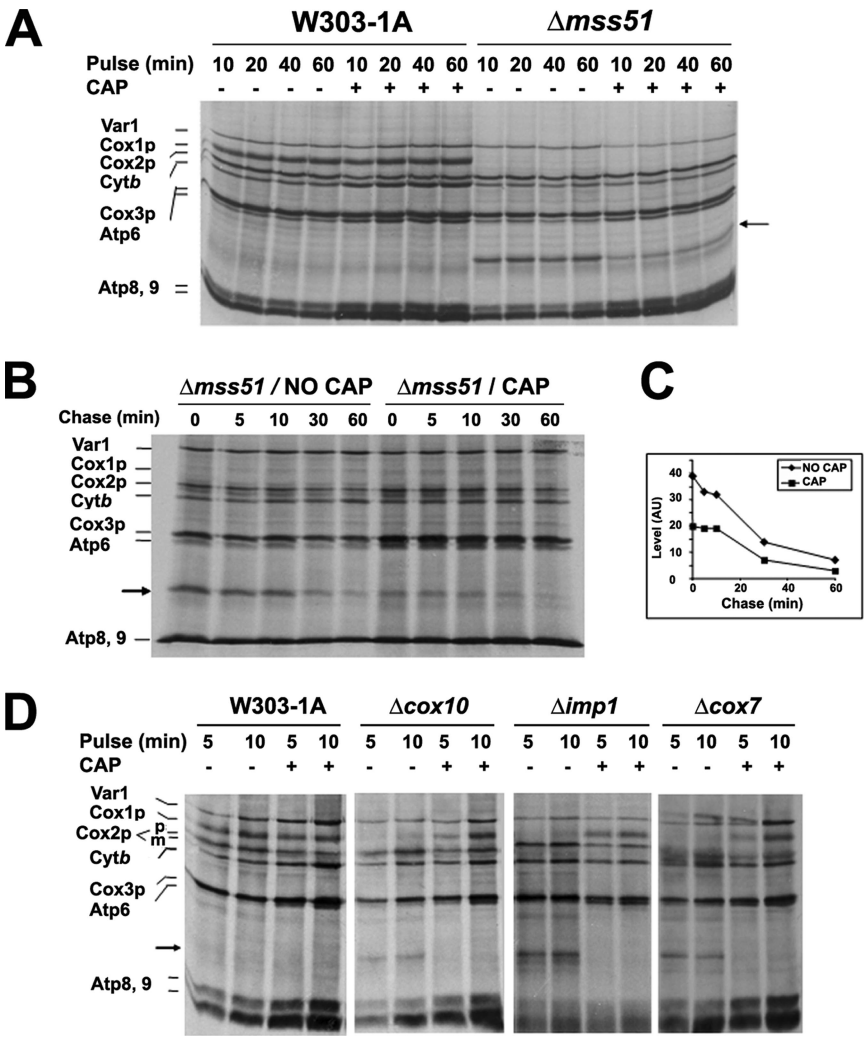

Figure 5. Synthesis and/or accumulation of mp15 are reduced in COX assembly mutants pretreated with chloramphenicol. (A) Effect of CAP pretreatment on in vivo labeling of mitochondrial gene products. Wild type (W303-1A) and the *mss1* null mutant (Δ*mss51*) were grown in YPGal. One-half of the cultures were further incubated at 30°C for 2 h in the presence of 2 mg/ml CAP. Cells were harvested and washed two times with a solution containing 40 mM potassium phosphate plus 2% galactose before labeling. Samples were removed after the indicated times of labeling and processed as in Figure 1. (B) Degradation of mp15 synthesized in cells pretreated or not with CAP. The *mss1* null mutant (Δ*mss51*) was incubated in the presence and absence of CAP as described in A. After harvesting and washing, cells were pulsed with [^{35}S]methionine for 10 min. Labeling was terminated by addition of 80 μmol of cold methionine and 12 μg/ml puromycin (0 time). Samples of the cultures were collected after the indicated times of incubation at 30°C and processed as described in Figure 1. (C) Quantification of mp15 degradation in B. The radiolabeled bands were detected and quantified with a PhosphorImager (GE Healthcare). The values (arbitrary units) were plotted against the time of chase. (D) Effect of CAP pretreatment on the mitochondrial protein synthesis pattern of *cox10*, *imp1*, and *cox7* null mutants. Cells were labeled as in A. Mitochondrial translation products are identified in the margin. Cox2p is not processed in Δ*imp1* mutant. The Cox2p precursor (pCox2p) in these strain migrates slower that the mature Cox2p (mCox2p). The functions affected in the different mutants are described in Table 1.

also assessed in different COX mutants. In agreement with our previous findings, the small amount of labeled Cox1p present in the mutants was stable for at least 2 h of chase (Barrientos *et al*., 2004; Figure 6C). In contrast, mp15 was almost completely degraded after the 2-h chase.

Turnover of mitochondrially synthesized COX subunits that do not enter the assembly pathway is a function of different proteases, including the m-AAA protease complex Yta10p/Yta12p (Arlt *et al*., 1996), which also acts as a chaperone for assembly of the mitochondrial respiratory chain complexes (Arlt *et al*., 1996). The protease function can be inactivated by the E559Q mutation in the active site of Yta10p without affecting the chaperone function of the complex (Arlt *et al*., 1996). The role of the Yta10p/Yta12p in turnover of mp15 was studied in *shy1/yta10* and a *cox11/yta10* double mutant harboring the *yta10*^{E559Q} allele. In vivo pulse-chase experiments of the double mutants revealed that mp15 is a substrate of Yta10p/Yta12p protease as a considerable amount of this novel protein was spared from degradation during the 1–2 h of chase in the background of the *yta10*^{E559Q} allele (Figure 6C).

Synthesis of mp15 Is Affected by the Intron Composition of COX1

W303Δ*mss51*, an *mss51* null mutant containing W303 mtDNA, had significantly more mp15 than the same mutant with D273 mtDNA (Figure 1). The only significant difference in the *COX1* genes of the two strains is the presence of additional introns in the gene of D273 mtDNA (Figure 7). In the absence of Mss51p, splicing of *COX1* pre-mRNA containing a particular combination of introns may result in the accumulation of a particular splicing intermediate that serves as an mRNA for the aberrant mp15 translation product.

To further ascertain the intron requirement for mp15 synthesis, mtDNA with different intron-containing *COX1* genes (Figure 7) were transferred to ρ^o derivatives of *mss51* and *shy1* null mutants. In vivo labeling of the mitochondrial products in the resultant strains showed that mp15 was detected as a prominent band in strains containing the mitochondrial genome of W303 but not D273 (Figure 1B). To confirm that the efficient production of mp15 was related to its *COX1* gene, mtDNA from Wi04, a strain with the same *COX1* introns as W303, was also transferred to the *mss51* and *shy1* null mutants. Synthesis of mp15 in both mutants was comparable with that seen in the same strains with the W303 mtDNA (data not shown). Based on the in vivo labeling results obtained with other *mss51* mutants harboring different *COX1* genes, however, it was not possible to relate the synthesis of mp15 to the absence of a particular intron, although the presence of aI1 and 5γ seems to be an absolute requirement (Figure 7).

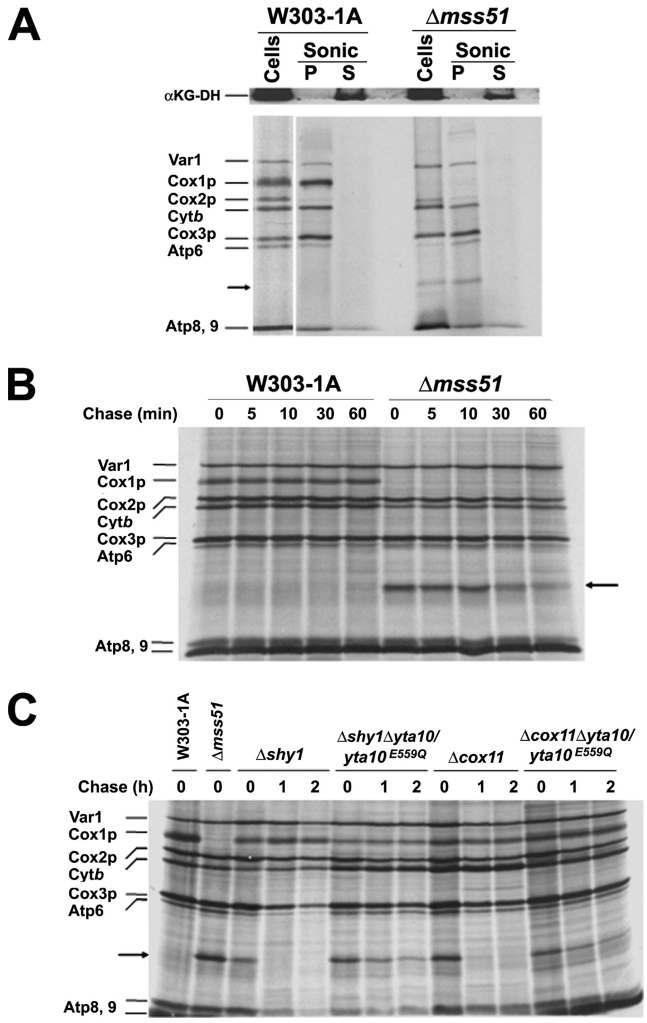

Figure 6. mp15 is a membrane protein whose degradation depends partially on a proteolytically active YTA10/YTA12 complex. (A) mp15 is a membrane protein. Wild type (W303-1A) and a mutant carrying a null allele of *mss51* were grown and labeled for 15 min at 30°C with [^{35}S]methionine in the presence of cycloheximide. Cells were subsequently converted to spheroplasts by digestion of the cell wall with zymolyase and submitted to sonic radiation. Samples were centrifuged 25,000 rpm, and the pellet (P) and supernatant (S) fractions were collected, TCA precipitated, and resuspended in loading buffer. The proteins were separated into a 17.5% PAGE and transferred to a nitrocellulose membrane. The membrane was both, exposed to x-ray film (bottom) and used for a Western blot that was probed with a polyclonal antibody against the matrix soluble protein α-ketoglutarate dehydrogenase (top). (B) mp15 is rapidly degraded after synthesis. Wild type (W303-1A) and a mutant carrying a null allele of *mss51* were grown and labeled for 15 min at 30°C with [^{35}S]methionine. Labeling was terminated by addition of 80 μmol of cold methionine and 12 μg/ml puromycin (0 time). Samples of the cultures were collected after the indicated times of incubation at 30°C and processed as in Figure 1. (C) Degradation of mp15 is partially mediated by the YTA10/YTA12 complex. The wild type strain W303-1A, the *shy1*, and *cox11* mutants, and the same mutants in which the endogenous wild-type *YTA10* gene had been substituted by the catalytically inactive *yta10*^{E559Q} mutant gene (Arlt *et al*., 1996) were labeled and chased for the indicated times as in A. Mitochondrial translation products are identified in the margin.

No synthesis of mp15 was seen in C199, a strain with D273 mtDNA and a point mutation in *mss51* causing partial loss of function (data not shown). This was also true of five other *mss51* point mutants in the same mitochondrial genetic background (data not shown). Substitution of D273 by W303 mtDNA in C199, however, allowed some limited synthesis of mp15. These results could suggest that expression of mp15 is at least partially dependent on the nuclear background or that the synthesis of mp15 is reduced in leaky mutants of *mss51*.

mp15 Is Translated from a Partially Processed COX1 Transcript

Synthesis of mp15 occurs in strains lacking Mss51p but not Pet309p, and additionally, it requires a *COX1* gene with a particular set of introns. Because processing and translation of *COX1* transcripts are interdependent events, splicing of some *COX1* introns being dependent on translation of intron-encoded maturases (Carignani *et al*., 1983, 1986), mp15 is likely to be derived from an incompletely processed *COX1* transcript. It was not excluded, however, that mp15 might be translated from an aberrantly spliced or partially degraded *COX1* transcript. To rule out these possibilities, we examined the *COX1* transcripts by Northern blot analysis of total mitochondrial RNAs of *mss51*, *pet309*, and *shy1* mutants (Figure 8). Even though the amount of mature *COX1* mRNA was reduced in the *mss51* mutant, as reported previously (Simon and Faye, 1984), the exon A5/intron aI5 probe revealed a similar pattern of *COX1* precursors in the *mss51* and *shy1* mutants (Figure 8A). No new small mRNA species were found in either strain (Figure 8A). The probe against the entire coding sequence of *COX1*, confirmed the presence both mature and partially processed transcripts in the *mss51* mutant with a *COX1* gene containing four or more introns (W303) (Figure 8B). In agreement with previous reports, neither mature nor precursor *COX1* RNAs were present in the *pet309* mutant because of their instability in this genetic background (Manthey and McEwen, 1995). Mature *COX1* mRNA was also detected in two point mutants of *mss51* in the D273 background, C199 and C283 (data not shown).

The presence of mature *COX1* mRNA in the *mss51* mutant suggests that there is sufficient translation of intron-encoded maturases for splicing of introns aI1, -2, and -3, although the possibility of enhanced self-splicing in the absence of Mss51p cannot be eliminated. As expected, normal levels of *COX1* mRNA are present in the *mss51* mutant with intronless mtDNA. The absence in this strain of mp15 also supports the notion that it is likely to be a translation product of a partially spliced *COX1* transcript.

Mss51p Binds to the 5′-UTR of COX1 mRNA

Mss51p has been conjectured to act on the *COXI* mRNA 5′-UTR to initiate translation and on the coding sequence to promote elongation (Decoster *et al*., 1990; Perez-Martinez *et al*., 2003). Because mp15 is only detected in the W303 and D273 mtDNA backgrounds, it is conceivable that the presence of a particular sequence(s) in the 5′-UTR of *COX1* of these strains could by-pass the requirement of Mss51p for translation initiation but not elongation. Under these circumstances, synthesis of Cox1p could be prematurely terminated.

The putative interaction/s of Mss51p with the 5′-UTR of *COX1* mRNA has not been characterized. We have used the Y3H depicted in Figure 9A to define both the RNA target(s) sequence and the Mss51p domain involved in the binding to the RNA. The constructs used as baits consisted of 460 base pairs 5′-UTR sequence (Manthey and McEwen, 1995) as well as different regions of the 5′-UTR with part of the first exon

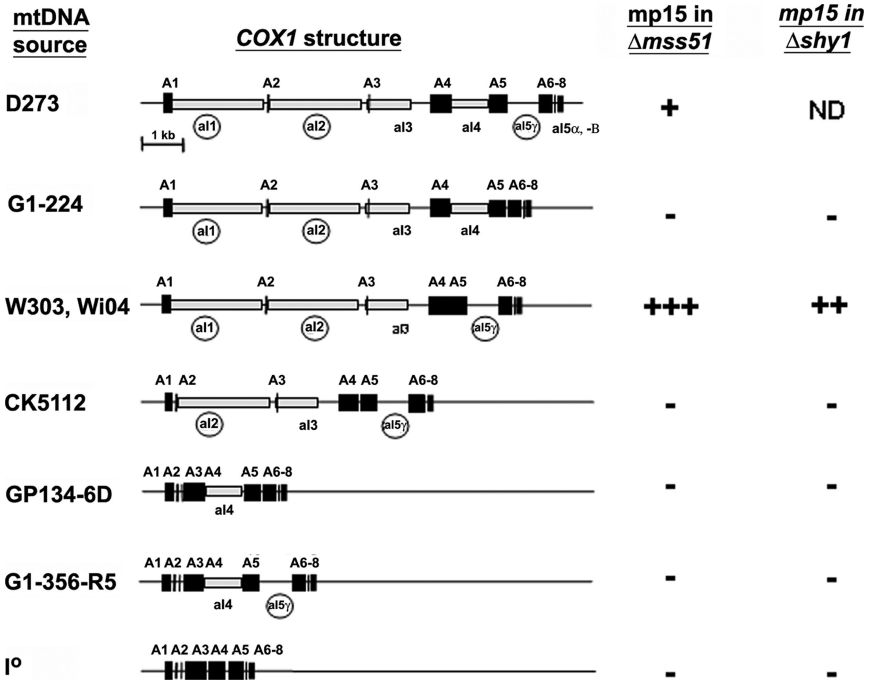

Figure 7. Synthesis of mp15 depends on the intron composition of the *COX1* gene. The figure represents the maps of *COX1* in different strains of yeast. Group II introns are circled. mtDNA containing *COX1* genes with different intron compositions (see details in Table 1) were transferred by cytoduction into to a *kar1* mutant (Conde and Fink, 1976) devoid of mtDNA (ρ^o). The different mitochondrial genomes were transferred from the *kar1* donor to ρ^o derivatives of *mss51* and *shy1* null mutants. To determine whether mp15 was synthesized in these strains, the mitochondrial gene products of the corresponding strains were labeled in vivo in the presence of cycloheximide and separated on a 17.5% polyacrylamide gel as in Figure 1. The last two columns on the right represent a summary of the results obtained.

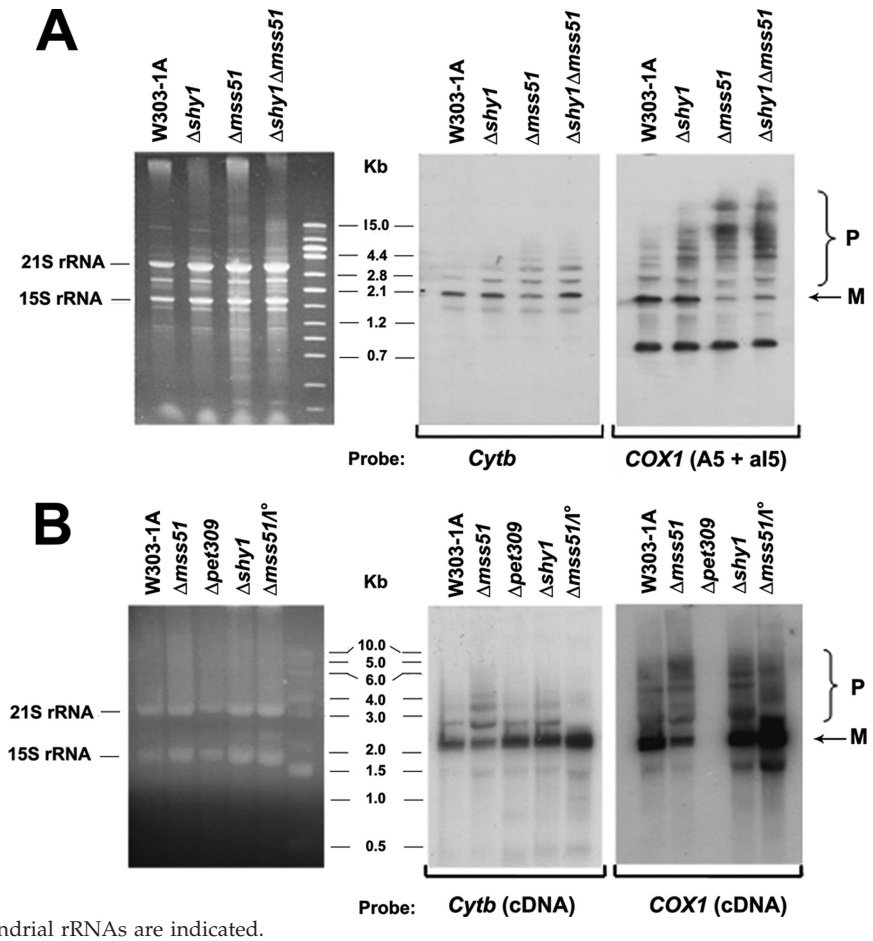

Figure 8. Mature and unprocessed *COX1* transcripts accumulate in *mss51* and *shy1* but not in *pet309* null mutants. (A) Mitochondrial RNA was extracted from mitochondria purified of the wild-type strain W303-1A, a *shy1* null mutant (Δ*shy1*), an mss51 null mutant (Δ*mss51*) and a double *shy/1mss51* mutant (Δ*shy1* Δ*mss51*). The RNA extracts were separated onto a 1% agarose gel, stained with ethidium bromide, photographed, and the RNAs blotted to a nylon membrane (Nytran, SuPerCharge; Whatman Schleicher and Schuell, Keene, NH). After cross-linking with UV light, the nylon membrane was prehybridized at 43°C with 125 μg of salmon sperm DNA in 5× SSC, 5× Denhardts, 0.5% SDS. The blotted RNAs were hybridized overnight at 43°C with probes containing exon 5 + intron 5 of *COX1* and exon 1 of *COB*. Both probes were labeled with [α-^{32}P]dATP by random priming (Feinberg and Vogelstein, 1983). (B) Mitochondrial RNA extracts were separated onto a denaturing 1.2% agarose gel, blotted to a nylon membrane and UV-light cross-linked. Northern blots of mitochondrial RNA of the wild-type W303-1A, the *mss51*, *pet309*, and *shy1* null mutants with a W303 mtDNA background (Figure 7A) and the *mss51* null mutant with an intronless mitochondrial genome were prehybridized at 42°C for 2 h with 1 μg of salmon sperm and hybridized overnight at 65°C in a solution containing 7% SDS, 1 μM EDTA, and 0.5 M Na_2PO_4 with probes containing the entire coding sequence of *COX1* or *COB*. The mature (M) and unprocessed (P) *COX1* transcripts are identified in the *margins*. The left panels in A–C shows the ethidium bromide-stained gels used for the Northern blots. The positions of the 15S and 21S mitochondrial rRNAs are indicated.

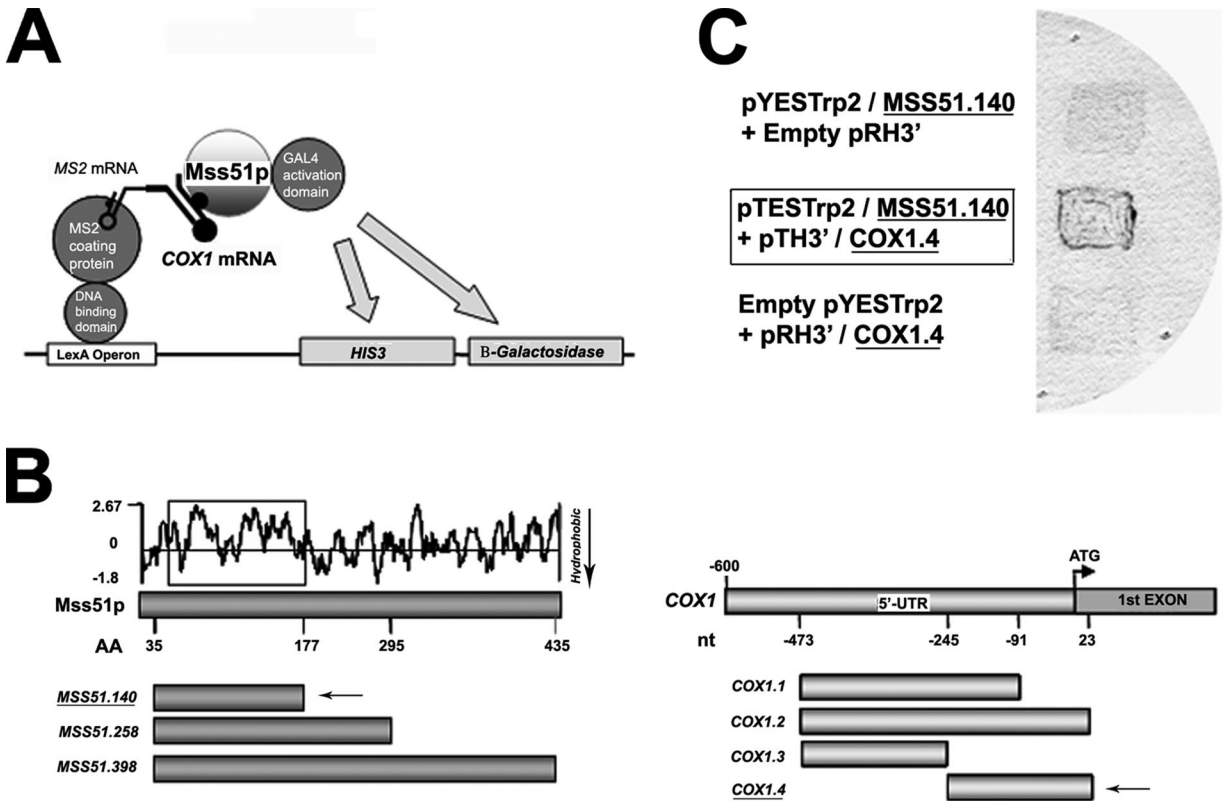

Figure 9. Mss51p binds to the 5′-UTR of *COX1* mRNA. (A) Y3H strategy used to explore the interaction of Mss51p with the 5′-UTR of *COX1* mRNA. (B) Diagram showing the regions of Mss51p (including a hydrophobicity map) and the *COX1* mRNA that were used to make the Y3H constructs. (C) A domain in the N′-terminus of Mss51p (Mss51.140; marked with a square in the hydrophobicity map in B) interacts with a domain in the 5′-UTR of *COX1* mRNA (Cox1.4; marked with an arrow in B). Activation of the β-galactosidase reporter gene detected as a blue color occurred only when both the protein prey and RNA bait were expressed.

of the *COX1* gene (Figure 9B). To ensure targeting of the Mss51p/Gal4 fusion proteins to the nucleus, a requirement in the Y3H system, the mitochondrial leader peptide of Mss51p was deleted. The mitochondrial import sequence of Mss51p determined by protein sequencing of Mss51p-GST purified from mitochondria consists of the N-terminal 35 residues encoded by the gene.

The results obtained with different *COX1* and *MSS51* constructs indicated an interaction between the MSS51-140 domain expressing the first 177 amino acids of the mature protein and a sequence located between nucleotides −245 and +23 of *COX1* mRNA (Figure 9C). This domain did not interact with the MSS51-398 construct, which should produce the entire Mss51p (data not shown), perhaps because folding of the membrane protein interferes with recognition of the bait. No interaction was detected between MSS51-140 and COX1.1 (data not shown), suggesting that the interacting mRNA domain is located between nucleotides −97 and +23, although we cannot exclude the possibility that the longer COX1.1, as in COX1.2, made the mRNA bait unsuitable for the assay. The MSS51–140 sequence encodes a hydrophilic domain, which probably protrudes into the mitochondrial matrix where it interacts with the 5′-UTR of *COX1* mRNA.

The region of the 5′-UTR of *COX1* that was ascertained to interact with Mss51p did not display any nucleotide variation in the mtDNAs of D273, W303, and the strain with the intronless gene.

DISCUSSION

Mss51p is a mitochondrial inner membrane protein that faces the matrix (Siep *et al*., 2000). Together with Pet309p, it interacts with the 5′-UTR of the *COX1* mRNA to initiate translation (Perez-Martinez *et al*., 2003). The yeast three-hybrid experiments reported here suggest that the target in the 5′-UTR of *COX1* mRNA could be within 245 nucleotides upstream of the initiation codon. The sequence in Mss51p that could interact with this activation site has been mapped to a hydrophilic region located in the N-terminal 177 residues of the mature protein.

As noted previously, *mss51* mutants lack Cox1p; unexpectedly, however, we found that they synthesize a novel 15-kDa protein. Several lines of evidence suggest that mp15 is translated from a *COX1* transcript. This protein is not present in *pet309* mutants in which initiation of *COX1* translation is blocked and which do not accumulate mature or precursor *COX1* mRNAs when this mitochondrial gene contains more than four introns (Manthey and McEwen, 1995). In addition, no mp15 is detected in an *mss51/pet309* double mutant or in a mutant with a large deletion in *COX1*.

Translation of a Cox1p-related protein in *mss51* mutants is at variance with previous observations that the interaction of Mss51p with the 5′-UTR of *COX1* is essential for translation of Cox1p. This was evident by the failure of an *mss51* mutant to express *ARG8*^{m} when the recoded gene was substituted for *COX1* and was fused to the normal 5′-UTR of *COX1* (Perez-Martinez *et al*., 2003). The possibility that the require-

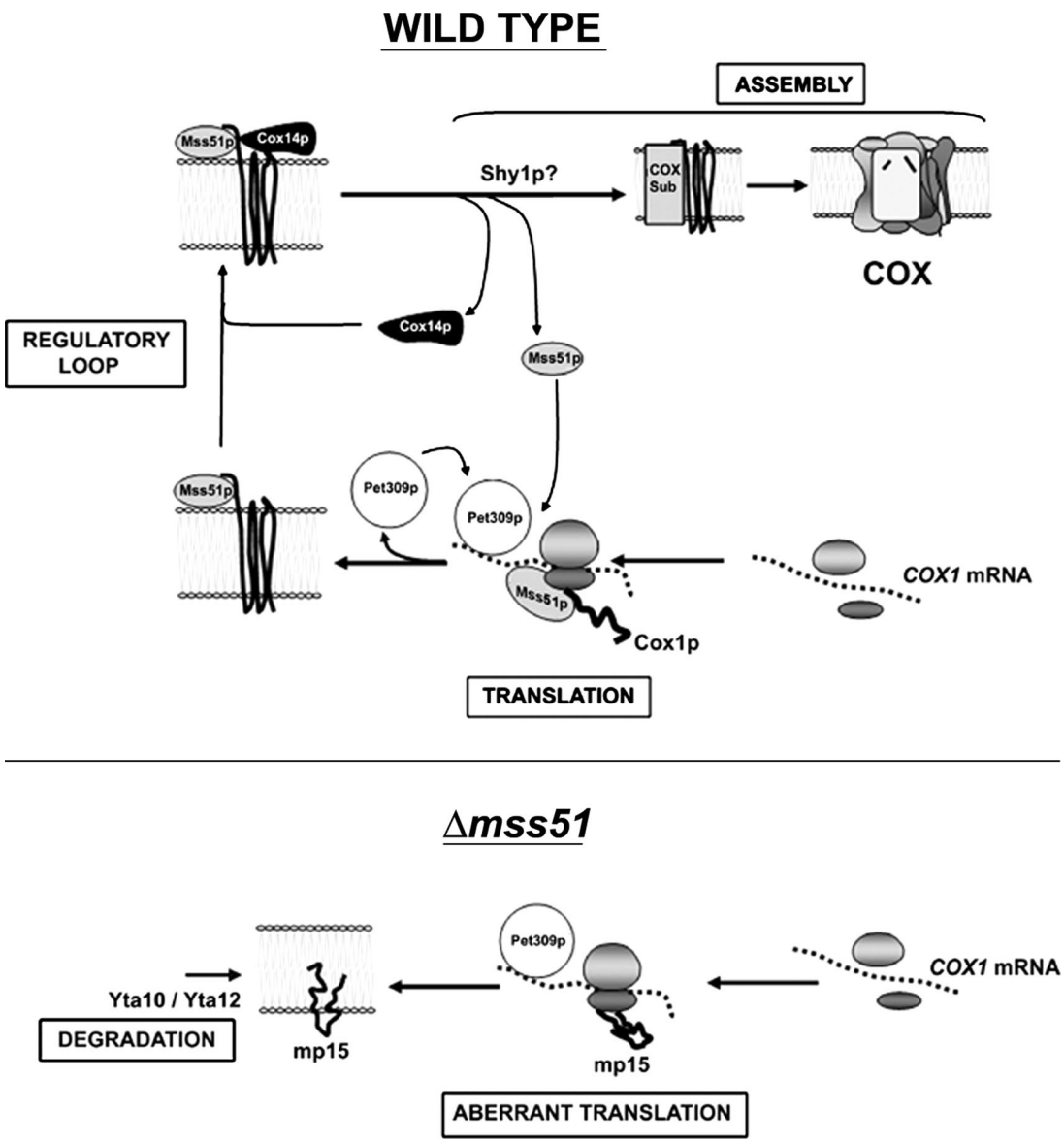

Figure 10. Model depicting the hypothetical roles of Mss51p in Cox1p translation and coupling to Shy1p-dependent COX assembly. Mss51p is required for translation of Cox1p by acting, together with Pet309p on the 5′-UTR of *COX1* mRNA. As shown before (Barrientos *et al*., 2004), Mss51p binds newly synthesized Cox1p forming a transient complex that is stabilized by Cox14p. A downstream event, maybe catalyzed by Shy1p, causing Cox1p to dissociate from the ternary complex makes Mss51p available for new rounds of translation. To simplify the model we have not included the action of Mss51p on the coding sequence of *COX1*, probably on Cox1p itself, interpreted as necessary for elongation of the nascent polypeptide (Perez-Martinez *et al*., 2003). In *mss51* mutants (Δ*mss51*) Mss51p-independent translation from alternate initiation sites in a *COX1* mRNA precursor occur generating a new polypeptide, mp15, which is proteolytically degraded.

ment for Mss51p for translation is by-passed in mp15-producing strains as a result of a polymorphism in the *COX1* 5′-UTRs has been excluded as no sequence differences were found in their Mss51p-interacting domain. These observations suggest that translation of mp15 is initiated from an ATG other than the normal *COX1* initiation codon. The dependence of mp15 expression on Pet309p, a protein implicated in translation initiation of *COX1* mRNA (Manthey and McEwen, 1995), may be related to the already mentioned absence of *COX1* transcripts in *pet309* mutants and therefore does not exclude the use of an alternate start codon. Whether translation of mp15 starts in an exon or intron is unclear, although an exonic ATG is more likely for several reasons. The partially overlapping peptide patterns of Cox1p and mp15 indicate the presence in the latter of some exon-encoded sequences. Because the reading frames of *COX1* introns are in register with the reading frames of their upstream but not downstream exons, a 15-kDa product could be expressed exclusively from an mRNA containing intron sequences but not from a reading frame initiated in an intron and encompassing the downstream exon.

Alternatively, mp15 could result from premature termination of translation of a *COX1* transcript. Recent evidence has shown that Mss51p acts on target/s within the coding sequence of the *COX1* mRNA or to the protein itself, to promote translational elongation and that in the absence of Mss51p, nascent Cox1p may block its own synthesis (Perez-Martinez *et al*., 2003). Translational regulation by nascent

chains has been reported previously (for review, see Tenson and Ehrenberg, 2002).

The mp15 polypeptide is most evident in *mss51* mutant containing mtDNA of W303, to a lesser extent in *mss51* mutants with D273 mtDNA, and it is not detected in strains with an intronless *COX1* or with combinations of introns other than those of W303 and D273. The appearance of novel mitochondrial translation products has been reported previously. For example, a *cox1* mutant expressing both Pet309p and Mss51p but defective in intron aI1 splicing was shown to produce a 90-kDa protein translated from the continuous reading frame encoded by the first exon and part of the first intron of *COX1*. This protein is post-translationally cleaved into two polypeptides of 20 and 68 kDa, the latter constituting the maturase encoded by intron aI1 (Carignani *et al*., 1983). In contrast, synthesis of mp15 depends on the complete absence of Mss51p or presence of only limiting amounts of the translationally competent protein. That mp15 is not detected in an *mss51* null mutant with intronless mtDNA supports the notion that this protein is translated from a *COX1* precursor transcript with one or more introns. The *COX1* genes of W303 and D273 contain all three group II introns (aI1, -2, and -5γ) and the group I intron aI3. The intron composition of *COX1* in D273 is similar to W303 except for the presence of group I intron aI4, which seems to suppress mp15 synthesis. It is also noteworthy that the only difference between the *COX1* genes of D273 and G1–224, which does not express mp15, is the absence in the latter of intron aI5γ. The lack of a clear correlation between the presence or absence of a particular intron and mp15 synthesis suggests that the overall intronic composition in the context of the *mss51* mutation may be crucial for the production or stability of the transcript corresponding to the mp15 mRNA.

Cox1p synthesis is regulated by downstream events occurring during assembly of the COX holoenzyme (Perez-Martinez *et al*., 2003; Barrientos *et al*., 2004). In our model, entry of newly translated Cox1p into the COX assembly pathway depends on its interaction with Mss51p and Cox14p (Figure 10). When engaged in this ternary complex, Mss51p is forestalled from initiating new rounds of Cox1p synthesis. Dissociation of Mss51p from the complex is envisioned to occur when Cox1p acquires its prosthetic groups or interacts with other COX subunits. This step may be catalyzed by Shy1p (Perez-Martinez *et al*., 2003; Barrientos *et al*., 2004). This is supported by the severe repression of Cox1p translation in mutants that are blocked in COX assembly (Barrientos *et al*., 2004), in which mp15 is also synthesized because in these strains the amount of Mss51p available for normal *COX1* mRNA translation is limited. The single exception are *cox14* mutants, in which normal amounts of Cox1p are synthesized and Mss51p is available for translation even in the absence of COX assembly (Barrientos *et al*., 2004). For simplicity, our model depicted in Figure 10 does not include the role of Mss51p on the coding sequence of *COX1* mRNA. As mentioned above, the interaction of Mss51p with Cox1p itself could be necessary for elongation of the nascent polypeptide and it could in addition regulate Cox1p synthesis (Perez-Martinez *et al*., 2003).

The regulatory model portrayed in Figure 10 is also supported by the results presented here. Synthesis of mp15 is Pet309p-dependent and Mss51p-independent, whereas synthesis of Cox1p requires both translational factors (Figure 10). Cox1p and mp15 synthesis are inversely related, suggesting that synthesis of Cox1p may suppress Mss51p-independent translation from alternate initiation sites by competing for Pet309p. Translation of mp15 in COX assembly mutants (not depicted in Figure 10 for simplicity) also points to the existence of a Cox14p-stabilized Mss51p-Cox1p complex. When trapped in this complex Mss51p is unavailable for translation of Cox1p. Mutations in *COX14*, either alone or in combination with mutations in other COX assembly factors (except *MSS51*), inhibit synthesis of mp15. This is consistent with the model as the instability of the Mss51p-Cox1p complex in the absence of Cox14p would be expected to make more Mss51p available for translation of Cox1p. The lack of an effect on mp15 synthesis of overexpression or deletion of *COX14* in an *mss51* null mutant, indicates that Cox14p does not directly repress Cox1p synthesis but rather does so indirectly by limiting Mss51p available for translation. Finally, incubation of cells in the presence of CAP before the [^{35}S]methionine pulse enhances synthesis of Cox1p in most COX assembly mutants. This may be explained by the synthesis of extra Mss51p during the CAP preincubation leading to enhanced translation of Cox1p, whereas having the opposite effect on mp15.

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REFERENCES

Arlt, H., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1996). The YTA10-12 complex, an AAA protease with chaperone-like activity in the inner membrane of mitochondria. Cell *85*, 875–885.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). *Saccharomyces cerevisiae*. In: Current Protocols in Molecular Biology, Vol. 2, Wiley, New York, 13.

Barrientos, A., Barros, M. H., Valnot, I., Rotig, A., Rustin, P., and Tzagoloff, A. (2002a). Cytochrome oxidase in health and disease. Gene *286*, 53–63.

Barrientos, A., Korr, D., and Tzagoloff, A. (2002b). Shy1p is necessary for full expression of mitochondrial *COX1* in the yeast model of Leigh's syndrome. EMBO J. *21*, 43–52.

Barrientos, A., Zambrano, A., and Tzagoloff, A. (2004). Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. EMBO J. *23*, 3472–3482.

Barros, M. H., Myers, A. M., Van Driesche, S., and Tzagoloff, A. (2006). *COX24* codes for a mitochondrial protein required for processing of the *COX1* transcript. J. Biol. Chem. *281*, 3743–3751.

Barros, M. H., Nobrega, F. G., and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. J. Biol. Chem. *277*, 9997–10002.

Breeden, L., and Nasmyth, K. (1985). Regulation of the yeast HO gene. Cold Spring Harb. Symp. Quant. Biol. *50*, 643–650.

Brown, N. G., Costanzo, M. C., and Fox, T. D. (1994). Interactions among three proteins that specifically activate translation of the mitochondrial *COX3* mRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *14*, 1045–1053.

Carignani, G., Groudinsky, O., Frezza, D., Schiavon, E., Bergantino, E., and Slonimski, P. P. (1983). An mRNA maturase is encoded by the first intron of the mitochondrial gene for the subunit I of cytochrome oxidase in *S. cerevisiae*. Cell *35*, 733–742.

Carignani, G., Netter, P., Bergantino, E., and Robineau, S. (1986). Expression of the mitochondrial split gene coding for cytochrome oxidase subunit I in *S. cerevisiae*: RNA splicing pathway. Curr. Genet. *11*, 55–63.

Carlson, C. G., Barrientos, A., Tzagoloff, A., and Glerum, D. M. (2003). *COX16* encodes a novel protein required for the assembly of cytochrome oxidase in *Saccharomyces cerevisiae*. J. Biol. Chem. *278*, 3770–3775.

Conde, J., and Fink, G. R. (1976). A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA *73*, 3651–3655.

Decoster, E., Simon, M., Hatat, D., and Faye, G. (1990). The *MSS51* gene product is required for the translation of the COX1 mRNA in yeast mitochondria. Mol. Gen. Genet. *224*, 111–118.

Faye, G., Kujawa, C., and Fukuhara, H. (1974). Physical and genetic organization of petite and grande yeast mitochondrial DNA. IV. In vivo transcription products of mitochondrial DNA and localization of 23 S. ribosomal RNA in petite mutants of *Saccharomyces cerevisiae*. J. Mol. Biol. *88*, 185–203.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6–13.

Fiori, A., Perez-Martinez, X., and Fox, T. D. (2005). Overexpression of the *COX2* translational activator, Pet111p, prevents translation of *COX1* mRNA and cytochrome c oxidase assembly in mitochondria of *Saccharomyces cerevisiae*. Mol. Microbiol. *56*, 1689–1704.

Glerum, D. M., Koerner, T. J., and Tzagoloff, A. (1995). Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase. J. Biol. Chem. *270*, 15585–15590.

Glerum, D. M., Muroff, I., Jin, C., and Tzagoloff, A. (1997). COX15 codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. J. Biol. Chem. *272*, 19088–19094.

Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996a). Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J. Biol. Chem. *271*, 14504–14509.

Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996b). SCO1 and *SCO2* act as high copy suppressors of a mitochondrial copper recruitment defect in *Saccharomyces cerevisiae*. J. Biol. Chem. *271*, 20531–20535.

Glerum, D. M., and Tzagoloff, A. (1997). Submitochondrial distributions and stabilities of subunits 4, 5, and 6 of yeast cytochrome oxidase in assembly defective mutants. FEBS Lett. *412*, 410–414.

Hell, K., Tzagoloff, A., Neupert, W., and Stuart, R. A. (2000). Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. J. Biol. Chem. *275*, 4571–4578.

Krause, K., Lopes de Souza, R., Roberts, D. G., and Dieckmann, C. L. (2004). The mitochondrial message-specific mRNA protectors Cbp1 and Pet309 are associated in a high-molecular weight complex. Mol. Biol. Cell *15*, 2674–2683.

Labouesse, M. (1990). The yeast mitochondrial leucyl-tRNA synthetase is a splicing factor for the excision of several group I introns. Mol. Gen. Genet. *224*, 209–221.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Manthey, G. M., and McEwen, J. E. (1995). The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. EMBO J. *14*, 4031–4043.

McEwen, J. E., Ko, C., Kloeckner-Gruissem, B., and Poyton, R. O. (1986). Nuclear functions required for cytochrome c oxidase biogenesis in *Saccharomyces cerevisiae*. Characterization of mutants in 34 complementation groups. J. Biol. Chem. *261*, 11872–11879.

Michaelis, U., Korte, A., and Rodel, G. (1991). Association of cytochrome *b* translational activator proteins with the mitochondrial membrane: implications for cytochrome *b* expression in yeast. Mol. Gen. Genet. *230*, 177–185.

Myers, A. M., Pape, L. K., and Tzagoloff, A. (1985). Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. EMBO J. *4*, 2087–2092.

Naithani, S., Saracco, S. A., Butler, C. A., and Fox, T. D. (2003). Interactions among *COX1*, *COX2*, and *COX3* mRNA-specific translational activator proteins on the inner surface of the mitochondrial inner membrane of *Saccharomyces cerevisiae*. Mol. Biol. Cell *14*, 324–333.

Nobrega, M. P., Bandeira, S. C., Beers, J., and Tzagoloff, A. (2002). Characterization of *COX19*, a widely distributed gene required for expression of mitochondrial cytochrome oxidase. J. Biol. Chem. *277*, 40206–40211.

Nobrega, M. P., Nobrega, F. G., and Tzagoloff, A. (1990). COX10 codes for a protein homologous to the ORF1 product of *Paracoccus denitrificans* and is required for the synthesis of yeast cytochrome oxidase. J. Biol. Chem. *265*, 14220–14226.

Perez-Martinez, X., Broadley, S. A., and Fox, T. D. (2003). Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. EMBO J. *22*, 5951–5961.

Rothstein, R. J. (1983). One-step gene disruption in yeast. Methods Enzymol. *101*, 202–211.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368–379.

Schiestl, R. H., and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. *16*, 339–346.

Seraphin, B., Boulet, A., Simon, M., and Faye, G. (1987). Construction of a yeast strain devoid of mitochondrial introns and its use to screen nuclear genes involved in mitochondrial splicing. Proc. Natl. Acad. Sci. USA *84*, 6810–6814.

Siep, M., van Oosterum, K., Neufeglise, H., van der Spek, H., and Grivell, L. A. (2000). Mss51p, a putative translational activator of cytochrome c oxidase subunit-1 (*COX1*) mRNA, is required for synthesis of Cox1p in *Saccharomyces cerevisiae*. Curr. Genet. *37*, 213–220.

Simon, M., and Faye, G. (1984). Steps in processing of the mitochondrial cytochrome oxidase subunit I pre-mRNA affected by a nuclear mutation in yeast. Proc. Natl. Acad. Sci. USA *81*, 8–12.

Smith, D., Gray, J., Mitchell, L., Antholine, W. E., and Hosler, J. P. (2005). Assembly of cytochrome-c oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme. J. Biol. Chem. *280*, 17652–17656.

Solans, A., Zambrano, A., and Barrientos, A. (2004). Cytochrome *c* oxidase deficiency: from yeast to human. Preclinica 2, 336–348.

Souza, R. L., Green-Willms, N. S., Fox, T. D., Tzagoloff, A., and Nobrega, F. G. (2000). Cloning and characterization of *COX18*, a *Saccharomyces cerevisiae* PET gene required for the assembly of cytochrome oxidase. J. Biol. Chem. *275*, 14898–14902.

Tenson, T., and Ehrenberg, M. (2002). Regulatory nascent peptides in the ribosomal tunnel. Cell *108*, 591–594.

Tiranti, V., *et al*. (1998). Mutations of *SURF-1* in Leigh disease associated with cytochrome c oxidase deficiency. Am. J. Hum. Genet. *63*, 1609–1621.

Towpik, J. (2005). Regulation of mitochondrial translation in yeast. Cell. Mol. Biol. Lett. *10*, 571–594.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996). The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. Science *272*, 1136–1144.

Tzagoloff, A., Akai, A., and Foury, F. (1976). Assembly of the mitochondrial membrane system XVI. Modified form of the ATPase proteolipid in oligomycin-resistant mutants of *Saccharomyces cerevisiae*. FEBS Lett. *65*, 391–395.

Tzagoloff, A., Akai, A., Needleman, R. B., and Zulch, G. (1975). Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. J. Biol. Chem. *250*, 8236–8242.

Tzagoloff, A., Capitanio, N., Nobrega, M. P., and Gatti, D. (1990). Cytochrome oxidase assembly in yeast requires the product of *COX11*, a homolog of the P. denitrificans protein encoded by ORF3. EMBO J. *9*, 2759–2764.

Tzagoloff, A., and Dieckmann, C. L. (1990). PET genes of *Saccharomyces cerevisiae*. Microbiol. Rev. *54*, 211–225.

Zhu, Z., *et al*. (1998). SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nat. Genet. *20*, 337–343.