

# Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p

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**The post-transcriptional role of Mss51p in mitochondrial gene expression is of great interest since *MSS51* mutations suppress the respiratory defect caused by *shy1* mutations. *SHY1* is a *Saccharomyces cerevisiae* homolog of human *SURF1*, which when mutated causes a cytochrome oxidase assembly defect. We found that *MSS51* is required for expression of the mitochondrial reporter gene *ARG8<sup>m</sup>* when it is inserted at the *COX1* locus, but not when it is at *COX2* or *COX3*. Unlike the *COX1* mRNA-specific translational activator *PET309*, *MSS51* has at least two targets in *COX1* mRNA. *MSS51* acts in the untranslated regions of the *COX1* mRNA, since it was required to synthesize Arg8p when *ARG8<sup>m</sup>* completely replaced the *COX1* codons. *MSS51* also acts on a target specified by the *COX1* coding region, since it was required to translate either *COX1* or *COX1::ARG8<sup>m</sup>* coding sequences from an ectopic *COX2* locus. Mss51p was found to interact physically with newly synthesized Cox1p, suggesting that it could coordinate Cox1p synthesis with insertion into the inner membrane or cytochrome oxidase assembly.**

**Keywords:** cytochrome *c* oxidase/mitochondrial DNA/translation/untranslated regions

## Introduction

The inability to assemble fully functional cytochrome *c* oxidase underlies several encephalomyopathic disorders in humans, caused in most cases by recessive autosomal mutations (Shoubridge, 2001). Genetic studies of cytochrome oxidase deficiencies in the yeast *Saccharomyces cerevisiae* provide a useful tool for both identifying relevant human genes and helping to elucidate their mechanisms of action (Shoubridge, 2001; Barrientos *et al.*, 2002a; Steinmetz *et al.*, 2002). For example, human *SURF1* mutations cause the cytochrome oxidase deficiency associated with Leigh's syndrome (Tiranti *et al.*, 1998; Zhu *et al.*, 1998), apparently by interfering with efficient assembly of the complex (Tiranti *et al.*, 1999). Mutations in the homologous yeast gene *SHY1* similarly reduce cytochrome oxidase activity (Mashkevich *et al.*, 1997) by causing a partial assembly defect (Nijtmans *et al.*, 2001), and reduced pulse labeling of Cox1p *in vivo* (Barrientos *et al.*, 2002b). Interestingly, missense mutations in the yeast nuclear gene *MSS51* can compensate for

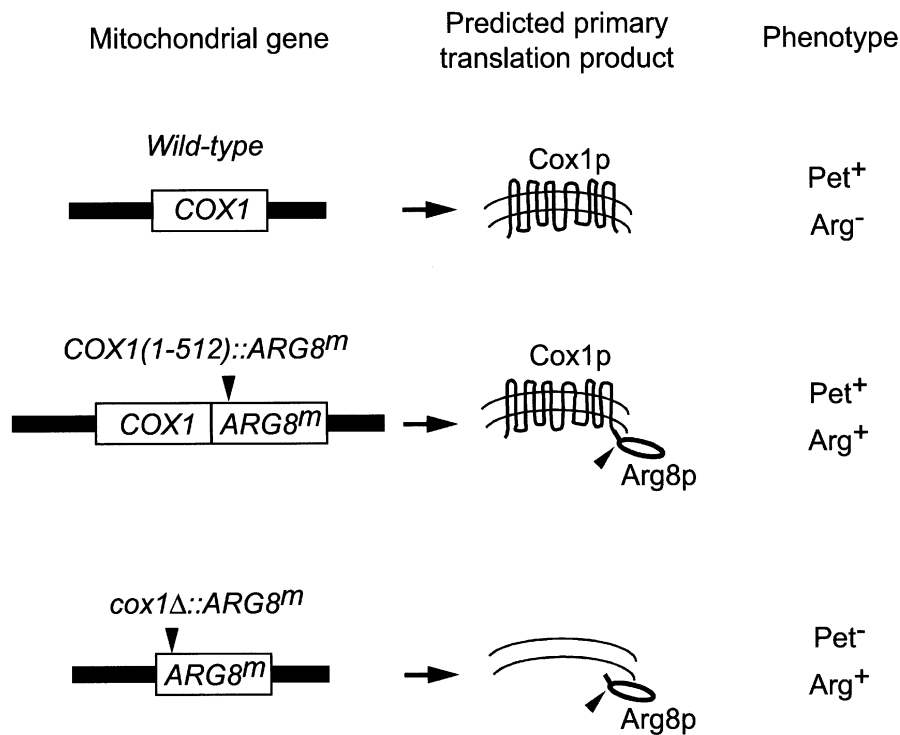
the loss of Shy1p (Barrientos *et al.*, 2002b). This finding suggests that Shy1p may have particular roles in the assembly and/or synthesis of cytochrome oxidase subunit I (Cox1p), since *mss51* null mutations fail to accumulate Cox1p (Decoster *et al.*, 1990).

Cox1p, the largest subunit of the enzyme, is coded in the mitochondrial DNA (mtDNA), synthesized on mitochondrial ribosomes and inserted into the inner membrane from the inside. Cox1p spans the mitochondrial inner membrane 12 times and is an integral part of the catalytic core of the enzyme (Tsukihara *et al.*, 1996). Insertion of Cox1p appears to be an early step in assembly of nuclear and mitochondrially encoded subunits of the complex (Lemaire *et al.*, 1998; Nijtmans *et al.*, 1998). In addition to genes encoding the enzyme subunits, biogenesis of yeast cytochrome oxidase requires a large set of nuclear-encoded genes whose products function in RNA processing, translation and assembly (Tzagoloff and Dieckmann, 1990; Grivell *et al.*, 1999). Several of these function chiefly in the expression of *COX1* (Kloeckener-Gruissem *et al.*, 1987; Pel *et al.*, 1992).

Among the nuclear genes required for cytochrome *c* oxidase activity are those that encode mRNA-specific translational activators for the *COX1*, *COX2* and *COX3* mRNAs (Fox, 1996). The translational activator proteins functionally interact with the 5'-untranslated leaders (5'-UTLs) of their target mRNAs. They are bound to the inner surface of the inner membrane (Fox, 1996; Manthey *et al.*, 1998; Green-Willms *et al.*, 2001), and are likely to play a role in localizing organellar translation (Sanchirico *et al.*, 1998). Interestingly, the *COX1* mRNA-specific activator protein Pet309p interacts with the *COX2* and *COX3* mRNA-specific activator proteins, suggesting that together they may co-localize Cox1p, Cox2p and Cox3p synthesis, promoting efficient assembly of the enzyme core (Naithani *et al.*, 2003).

The nuclear gene *MSS51* previously has been suggested to encode another mRNA-specific translational activator for the *COX1* mRNA (Decoster *et al.*, 1990; Siep *et al.*, 2000). *MSS51* was first identified as a gene required for the processing of introns in the *COX1* pre-mRNA (Faye and Simon, 1983). However, *mss51* mutations did not affect the level of *COX1* mRNA in strains lacking mitochondrial introns, but prevented Cox1p pulse-labeling and accumulation, suggesting a role in translation (Decoster *et al.*, 1990; Siep *et al.*, 2000). While Mss51p is associated with the mitochondrial inner membrane (Siep *et al.*, 2000), its mechanism of action remains unclear.

We have studied here the role of Mss51p in *COX1* expression and its targets of action using mitochondrial gene rearrangements and the mitochondrial reporter gene *ARG8<sup>m</sup>* (Steele *et al.*, 1996). This reporter encodes a matrix-localized biosynthetic enzyme normally specified by the nuclear gene *ARG8*, and allows us to look directly at



**Fig. 1.** *ARG8<sup>m</sup>* reporter constructs at *COX1*. The mitochondrial genes, predicted translation products, and arginine and respiratory phenotypes of strains bearing wild-type nuclear genomes are indicated. The open boxes represent the *COX1* and *ARG8<sup>m</sup>* structural genes. The *COX1* 5'-UTL and 3'-UTR are indicated as solid boxes. The black triangles indicate the processing site for the pre-Arg8p matrix-targeting signal. In the *COX1(1-512)::ARG8<sup>m</sup>* construct, the *ARG8<sup>m</sup>* gene was fused in-frame to the complete, 512 *COX1* codons, such that the *COX1* stop codon was replaced by the initial methionine of the *ARG8<sup>m</sup>* gene. In the *cox1Δ::ARG8<sup>m</sup>* construct, the *ARG8<sup>m</sup>* gene replaced the *COX1* codons, and was precisely fused to the *COX1* 5'-UTL and 3'-UTR.

mitochondrial translation by scoring growth of yeast in the absence of arginine and accumulation of the reporter protein. Our results confirm that Mss51p is specifically required for *COX1* mRNA translation, but its mechanism is clearly distinct from that of Pet309p and the other known mRNA-specific translational activators.

## Results

### ***MSS51* is required for expression of the *ARG8<sup>m</sup>* mitochondrial reporter inserted at *COX1***

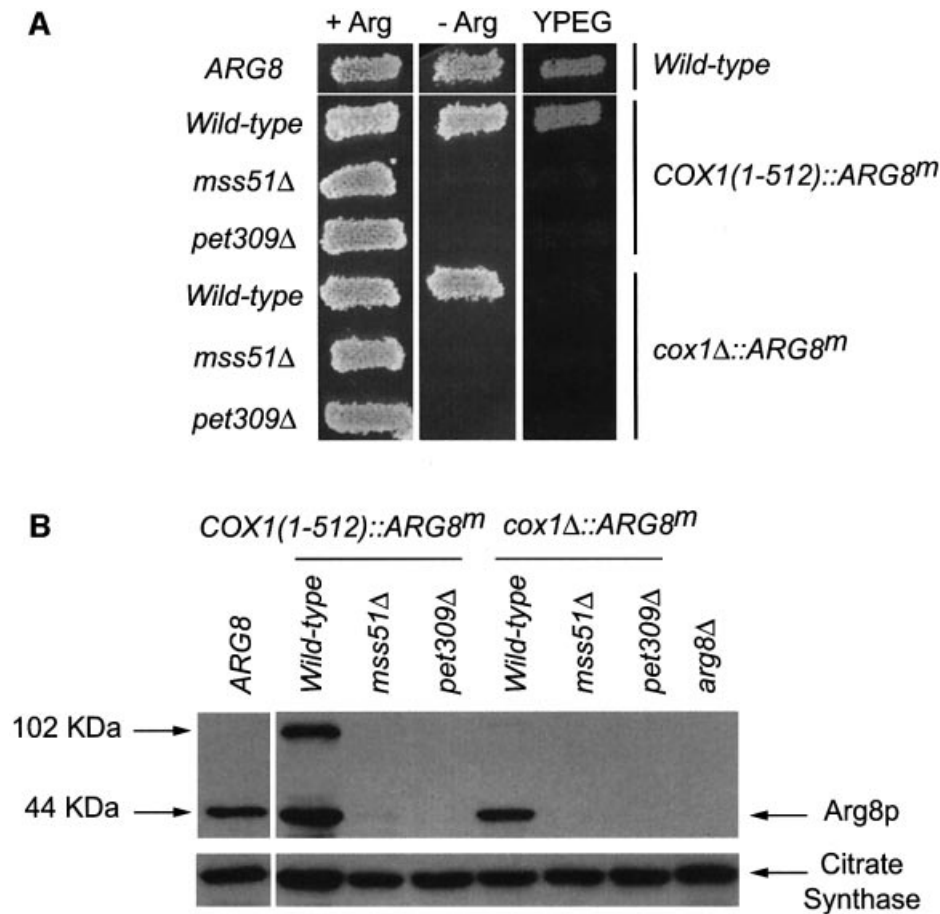
*mss51* mutations strongly reduce incorporation of radioactive label into Cox1p *in vivo* without affecting the level of intronless *COX1* mRNA (Decoster *et al.*, 1990; Siep *et al.*, 2000). These data suggest a defect in translation, but cannot rule out the possibility that Cox1p becomes highly unstable in the absence of Mss51p. Indeed, the fact that *MSS51* mutations could suppress the assembly defect of *shy1* mutants (Barrientos *et al.*, 2002b) suggested that Mss51p could be involved in assembly. We therefore reinvestigated the role of *MSS51* in *COX1* expression by inserting the mitochondrial reporter gene *ARG8<sup>m</sup>* (Steele *et al.*, 1996) at the end of the 512 codon intronless form of the *COX1* coding sequence [*COX1(1-512)::ARG8<sup>m</sup>*] (Figure 1).

The *ARG8<sup>m</sup>* product is a soluble biosynthetic enzyme normally located in the matrix and whose activity is unaffected by *mss51* mutations. Since the *ARG8<sup>m</sup>* sequence used here specifies the cleavage site for removal of the matrix-targeting signal of pre-Arg8p, accumulation

of processed Arg8p should not be affected by the instability of Cox1p fused upstream of it. We have previously shown that an unstable variant of Cox2p fused upstream of Arg8p did not affect Arg8p stability (Bonney *et al.*, 2001). Thus Arg8p provides a readout of *COX1* gene expression at the level of translation.

The *COX1(1-512)::ARG8<sup>m</sup>* gene was inserted into  $\rho^+$  mtDNA and the respiratory and Arg phenotypes of the resulting strain were examined (Figure 2A). In a wild-type nuclear background, this chimeric mitochondrial gene supported normal growth on both non-fermentable carbon sources and medium lacking arginine. However, an *mss51Δ*, *COX1(1-512)::ARG8<sup>m</sup>* strain could not grow on either a non-fermentable carbon sources or a medium lacking arginine, confirming that *MSS51* is required for synthesis of Cox1p (Figure 2A). As expected, a *pet309Δ*, *COX1(1-512)::ARG8<sup>m</sup>* strain was also both Pet<sup>-</sup> and Arg<sup>-</sup> (Figure 2A). Similar results were obtained with an intron-bearing *COX1(1-512)::ARG8<sup>m</sup>* construct derived from strain D273-10B (data not shown).

We also created strains in which *ARG8<sup>m</sup>* completely replaced the *COX1* coding sequence (*cox1Δ::ARG8<sup>m</sup>*) (Figure 1). In a wild-type nuclear background, the *cox1Δ::ARG8<sup>m</sup>* mitochondrial gene supported Arg<sup>+</sup> growth (Figure 2A). Both the *mss51Δ*, *cox1Δ::ARG8<sup>m</sup>* strain and the *pet309Δ*, *cox1Δ::ARG8<sup>m</sup>* strain were Arg<sup>-</sup>, indicating that *COX1* 5'- and 3'-untranslated regions (UTRs) cause translation to be dependent upon Pet309p, as expected, but also on Mss51p. Pet309p accumulation was not prevented by an *mss51Δ* mutation (data not shown), suggesting, but



**Fig. 2.** *MSS51* is required for translation of *ARG8<sup>m</sup>* when it is inserted at *COX1*. (A) Growth phenotypes. Relevant nuclear and mitochondrial genotypes are indicated on the left and right side of the panel, respectively. Yeast cells were grown on YPD and replica plated to glucose minimal medium containing (+Arg) or lacking (–Arg) arginine, and complete non-fermentable medium containing ethanol and glycerol (YPEG) as indicated. Cells carrying the nuclear-encoded *ARG8* gene were used for arginine growth comparison (*ARG8*). Cells were grown for 2 days at 30°C. (B) Steady-state accumulation of the reporter Arg8p. A 25μg aliquot of total mitochondrial proteins was separated by 12.5% SDS–PAGE, and the western blot was probed with anti-Arg8p antibody. The membrane was stripped and re-probed with antibody against citrate synthase as a loading control. An *arg8* null mutant (*arg8Δ*) was used as a negative control. Strain details are given in the Supplementary data.

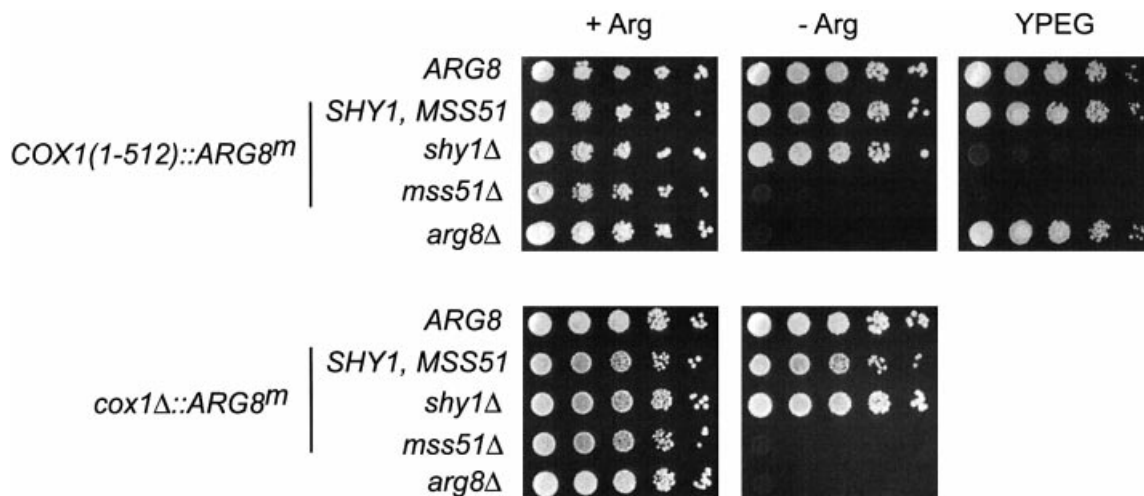
not demonstrating, that the two proteins may function independently. *mss51Δ* mutations specifically prevented expression of *ARG8<sup>m</sup>* inserted at *COX1*. *ARG8<sup>m</sup>* expression was not affected by *mss51Δ* when it was inserted in place of the *COX2* or *COX3* coding sequences (Supplementary figure 1S, available at *The EMBO Journal Online*).

The results of western blot analysis of mitochondrial protein extracts confirmed our conclusions based on growth phenotypes (Figure 2B). Mitochondria bearing the intronless *COX1(1–512)::ARG8<sup>m</sup>* gene contained processed 44 kDa Arg8p corresponding to the wild-type protein, and a 102 kDa species corresponding in size to the unprocessed Cox1p–preArg8p fusion protein. Mitochondria from the *cox1Δ::ARG8<sup>m</sup>* strain contained a band corresponding to the mature 44 kDa Arg8p. In addition, a band with a size close to 102 kDa was present, although its origin is uncertain. Levels of Arg8p were dramatically reduced in *mss51Δ* and *pet309Δ* mitochondria containing either the *COX1(1–512)::ARG8<sup>m</sup>* or *cox1Δ::ARG8<sup>m</sup>* reporter genes. (Overexposure revealed the presence of low levels of the 44 kDa species in *cox1Δ::ARG8<sup>m</sup>* mitochondria.) Similar results were obtained when the intron-containing version of the *COX1(1–512)::ARG8<sup>m</sup>*

gene was analyzed (data not shown). As expected, the levels of *ARG8<sup>m</sup>* mRNA in the *mss51Δ* mutants bearing the intronless *COX1(1–512)::ARG8<sup>m</sup>* gene or the *cox1Δ::ARG8<sup>m</sup>* gene were equivalent to those in otherwise wild-type strains (data not shown).

We also investigated the effect of a *shy1Δ* mutation on expression of the *COX1(1–512)::ARG8<sup>m</sup>* and *cox1Δ::ARG8<sup>m</sup>* reporters. The *shy1Δ* mutation should not block synthesis of the Cox1p–preArg8p reporter protein if Shy1p is only required for assembly. Consistent with this expectation, the *shy1Δ*, *COX1(1–512)::ARG8<sup>m</sup>* strain exhibited an Arg<sup>+</sup> phenotype, but failed to grow robustly on non-fermentable carbon sources (Figure 3). This result confirmed that the *COX1(1–512)::ARG8<sup>m</sup>* reporter produces an Arg<sup>+</sup> phenotype if translated, even if the Cox1p moiety fails to assemble. Similarly, the *shy1Δ* mutation had no effect on the Arg<sup>+</sup> phenotype of a *cox1Δ::ARG8<sup>m</sup>* strain (Figure 3). Western analysis confirmed that *shy1Δ* had no effect on accumulation of the Arg8p reporter protein encoded by *COX1(1–512)::ARG8<sup>m</sup>* or *cox1Δ::ARG8<sup>m</sup>* (unpublished results).

Taken together, these data demonstrate that *MSS51* is required for the synthesis of Arg8p when *ARG8<sup>m</sup>* is at the



**Fig. 3.** The *shy1Δ* mutation does not affect the expression of *ARG8<sup>m</sup>* in the *COX1(1-512)::ARG8<sup>m</sup>* and *cox1Δ::ARG8<sup>m</sup>* mitochondrial genes. Ten-fold serial dilutions of cells bearing the *COX1(1-512)::ARG8<sup>m</sup>* or *cox1Δ::ARG8<sup>m</sup>* mitochondrial alleles were spotted on glucose minimal medium containing (+Arg) or lacking (–Arg) arginine, or complete ethanol/glycerol medium (YPEG), and incubated for 3 days at 30°C. In addition, a strain that expressed the nuclear-encoded *ARG8* gene (*ARG8*) and an *arg8* null mutant (*arg8Δ*) were included. Strain details are given in the Supplementary data.

*COX1* locus. Moreover, the fact that the expression of the reporter in the strain carrying the *cox1Δ::ARG8<sup>m</sup>* gene is dramatically reduced in the *mss51Δ* mutant indicates that targets of *MSS51* action must be located in the UTRs of the *COX1* mRNA, since this reporter gene lacks the coding region of *COX1* yet requires *MSS51* for expression.

#### ***MSS51* function is not bypassed by placing the *COX3* mRNA 5'-UTL on the *COX1* mRNA**

Respiratory deficiency caused by the absence of the previously characterized mRNA-specific translational activators, such as *PET309*, can be phenotypically suppressed by mitochondrial DNA rearrangements that fuse the affected mitochondrial coding sequence to a mitochondrial mRNA 5'-UTL containing the target of another activator (Fox, 1996). Thus, *pet309Δ* mutations are suppressed in strains containing chimeric *COX1* mRNAs lacking introns that bear a 5'-UTL from either the *COB* or *COX3* mRNA (Manthey, 1995; Manthey and McEwen, 1995). If the only function of *MSS51* were to work together with *PET309* to provide translational activation through the *COX1* mRNA 5'-UTL, then *mss51* mutations should be bypassed in strains containing the same chimeric mRNAs.

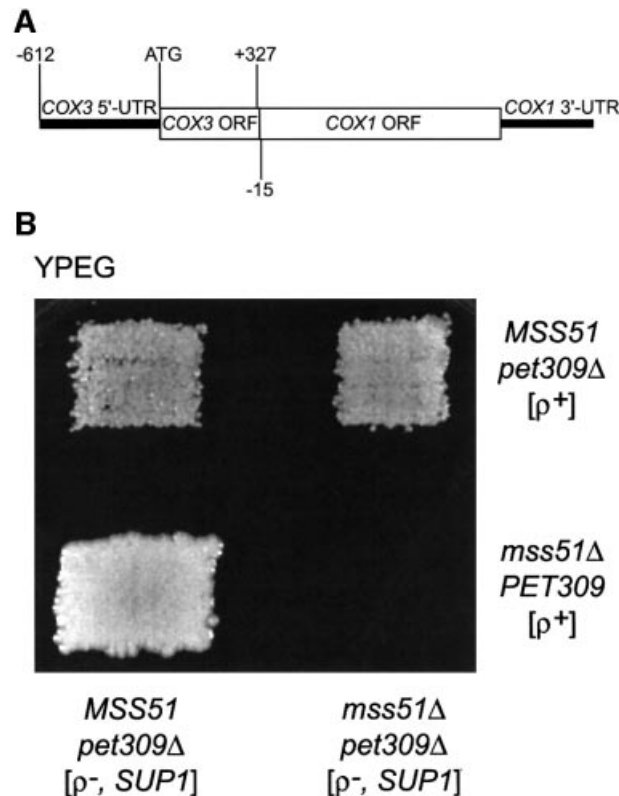
We tested the ability of a mtDNA rearrangement, previously selected to bypass *pet309Δ* mutations, to bypass an *mss51* deletion by generating *mss51Δ* cells containing both wild-type  $\rho^+$  mtDNA and a  $\rho^-$  mtDNA bearing the chimeric *COX1* gene in a heteroplasmic state. The mitochondrial *pet309Δ* bypass suppressor *SUP1* encodes a chimeric mRNA with the *COX3* mRNA 5'-UTL and part of the *COX3* coding sequence fused to the *COX1* coding sequence and *COX1* mRNA 3'-UTR (Manthey, 1995) (Figure 4A). We constructed an *mss51Δ*, *pet309Δ* strain carrying the *SUP1*,  $\rho^-$  genome and mated it to an *mss51Δ*, *PET309* strain bearing wild-type  $\rho^+$  mtDNA to generate *mss51Δ* homozygous cells heteroplasmic for the mtDNAs. These cells failed to respire, indicating that the presence of the *COX3* 5' leader on the chimeric mRNA

did not allow *COX1* expression in the absence of *Mss51p* (Figure 4B). *MSS51* cells homozygous for *pet309Δ* were respiratory competent as expected (Manthey, 1995). Thus, *MSS51* does not simply work in conjunction with *PET309* to activate translation through the mRNA 5'-UTL.

#### ***MSS51* function is not bypassed by replacing both the *COX1* mRNA 5'-UTL and 3'-UTR with those of the *COX2* mRNA**

If *MSS51* acted only through the *COX1* mRNA 3'-UTR or both the 5'-UTL and the 3'-UTR of the *COX1* mRNA, then replacement of both UTRs could bypass the requirement for *MSS51*. We therefore asked whether the *COX1* coding sequence could be expressed in the absence of *Mss51p* from a chimeric mRNA in which both the *COX1* mRNA 5'-UTL and 3'-UTR were replaced by the 5'-UTL and 3'-UTR derived from the *COX2* mRNA. This construct contains both the *COX2* promoter, 54 bp upstream of *COX1*, and the *COX2* dodecamer sequence specifying the 3' end of the mRNA, 75 bp downstream of *COX1* (Dieckmann and Staples, 1994; Mireau *et al.*, 2003). We integrated this chimeric gene ectopically into  $\rho^+$  *cox1Δ::ARG8<sup>m</sup>* mtDNA at a site 295 bp upstream of *COX2* (Figure 5A), where we know that inserted sequences do not prevent respiratory function (Thorsness and Fox, 1993).

A strain bearing this mtDNA and a wild-type nuclear genome was respiratory competent, albeit with a less than wild-type growth rate on non-fermentable medium, indicating functional expression of the chimeric *COX1* gene (Figure 5B). It was also Arg<sup>+</sup> due to expression of *cox1Δ::ARG8<sup>m</sup>*. As expected, a strain bearing this mtDNA and a *pet309Δ* mutation could not express the *cox1Δ::ARG8<sup>m</sup>* reporter, but was respiratory competent, indicating that the chimeric *COX1* mRNA could be translated in the absence of *Pet309p*. In contrast, a strain bearing this mtDNA and an *mss51Δ* mutation exhibited tight non-respiratory as well as Arg<sup>-</sup> phenotypes (Figure 5B). Therefore, replacement of both the 5'-UTL



**Fig. 4.** *MSS51* function cannot be bypassed by a *pet309Δ* suppressor. (A) Representation of the mtDNA rearrangement termed *SUP1* (Manthey, 1995) that allows *COX1* expression in a *pet309Δ* background. *SUP1* arose from a recombination event between the *COX3* locus and the *COX1* 5'-UTL sequence. The resulting chimeric gene has the entire *COX3* 5'-UTL sequence and the first 327 nucleotides of the *COX3* coding region fused to 15 nucleotides of the *COX1* 5'-UTR and the rest of *COX1*. (B) Haploid cells containing a  $\rho^-$  mtDNA bearing the *SUP1* gene were patched in vertical stripes. Their relevant nuclear genotypes are indicated. Cells containing wild-type,  $\rho^+$  mitochondria were patched on horizontal stripes. Their relevant nuclear genotypes are as indicated. The stripes were cross-printed on YPD medium and allowed to mate. The plate was then printed on non-fermentable YPEG medium and incubated for 5 days at 30°C. Strain details are given in the Supplementary data.

and 3'-UTR of the *COX1* mRNA with those of the *COX2* mRNA failed to bypass the requirement for Mss51p in *COX1* expression. The failure to respire reflects a block in *COX1* expression, since *mss51* mutations do not affect expression of other mitochondrial genes (Decoster *et al.*, 1990; Siep *et al.*, 2000) (Supplementary figure 1S.)

We conclude from these data that Mss51p must act on a target or targets encoded within the *COX1* coding sequence to allow functional expression of *COX1*, in addition to and independently of its role, demonstrated above, in working through the *COX1* mRNA UTRs to promote translation of the reporter.

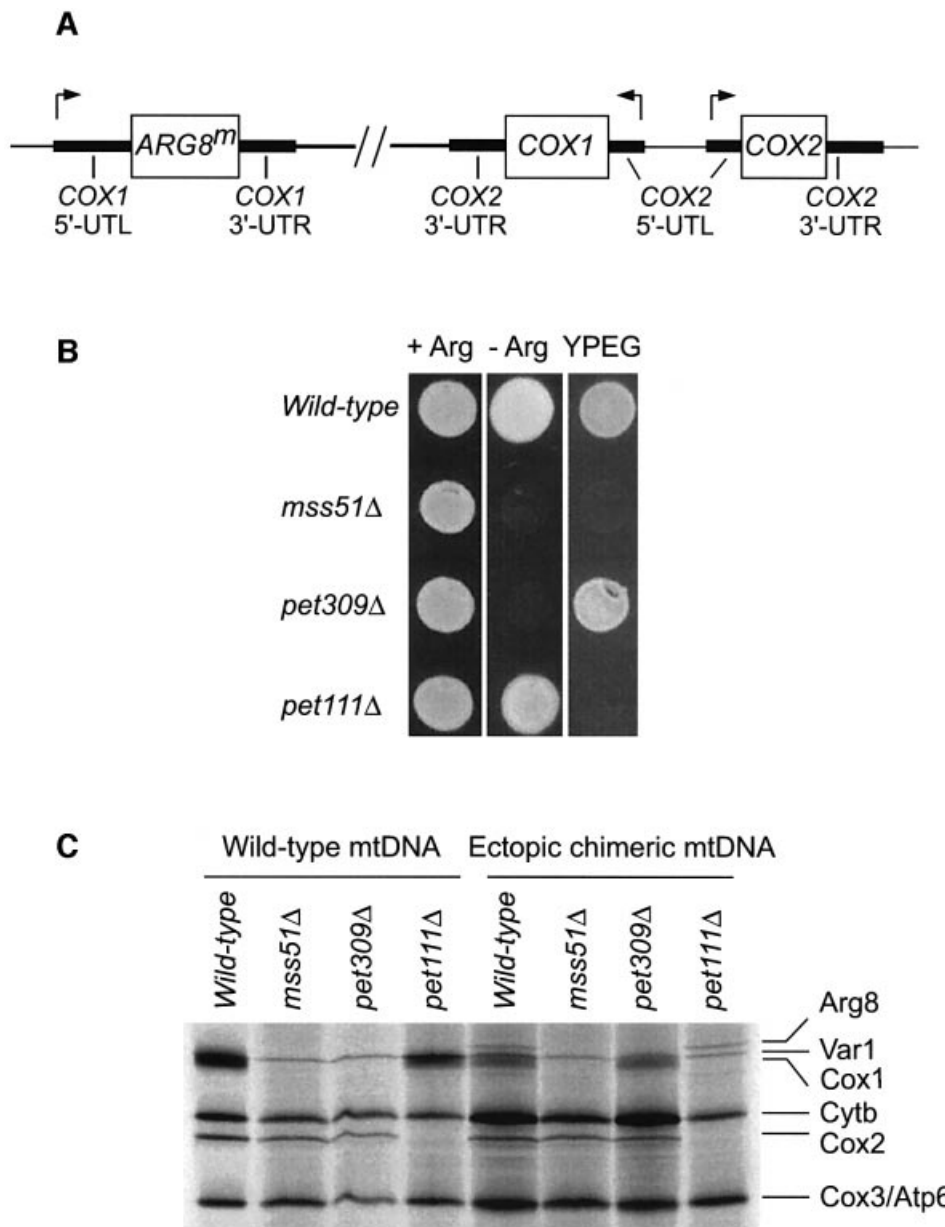
#### ***Mss51p* action on a target specified by the *COX1* coding sequence promotes translation**

While the experiments described above demonstrate the role of Mss51p in promoting translation of reporter mRNAs bearing *COX1* mRNA UTRs, it remained possible that Mss51p could play a different role in promoting expression of the *COX1* coding sequence itself when this coding sequence is flanked by *COX2* UTRs. For example, Mss51p could interact both with *COX1* mRNA UTRs to promote translation, and with nascent Cox1p itself to stabilize the protein during cytochrome oxidase assembly.

To investigate the action of Mss51p on the *COX1* coding sequence, we first examined pulse-labeled mitochondrial translation products from strains bearing the

ectopic *COX1* gene (Figure 5A), in comparison with strains with wild-type mtDNA. In strains with wild-type mtDNA, *mss51Δ* and *pet309Δ* mutations completely prevented labeling of Cox1p, while a *pet111Δ* mutation, which removes the *COX2* mRNA-specific translational activator, only reduced Cox1p labeling while eliminating Cox2p, as previously observed (Poutre and Fox, 1987) (Figure 5C). In a strain bearing the ectopic *COX1* chimeric gene and a wild-type nuclear genome, Cox1p labeling was reduced relative to a true wild-type, but was easily detectable. As expected, translation of the chimeric mRNA copied from this ectopic gene was not affected by the *pet309Δ* mutation but was eliminated by the *pet111Δ* mutation (Figure 5C). Labeling of Cox1p translated from the chimeric mRNA was reduced to undetectable levels by the *mss51Δ* mutation, suggesting that Mss51p acts at the level of *COX1* codon translation, even if the *COX1* codons are flanked by the *COX2* 5'-UTL and 3'-UTR (Figure 5C).

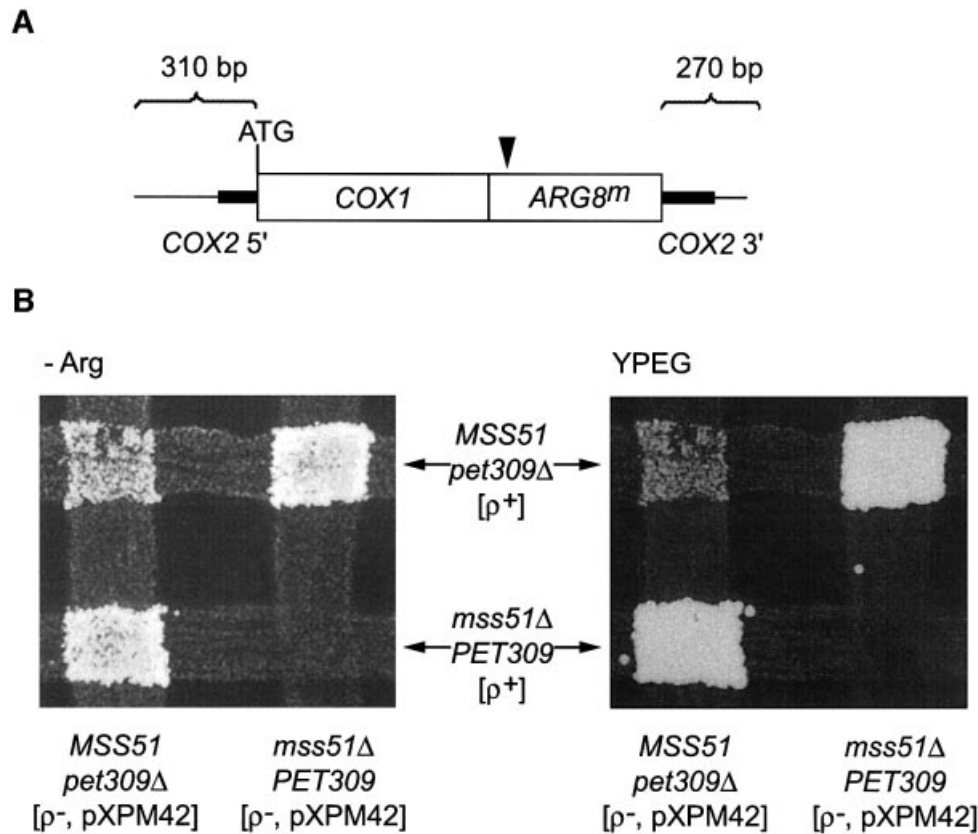
To confirm that Mss51p is required for translation of the chimeric *COX1* mRNA bearing the *COX2* 5'-UTL and 3'-UTR, we fused the *ARG8<sup>m</sup>* reporter to its *COX1* coding sequence and examined Arg8p expression. For reasons noted above, Arg8p expression from this chimeric gene provides a readout of *COX1* gene expression at the level of translation that is independent of the fate of the encoded Cox1p.



**Fig. 5.** *MSS51* is required to express the *COX1* coding sequence when it is fused to the *COX2* 5'-UTL and 3'-UTR at an ectopic locus. **(A)** Schematic representation of mtDNA bearing the ectopic chimeric *COX1* gene. The *COX1* structural gene is flanked by 73 and 119 bp encoding the *COX2* 5'-UTL and 3'-UTR, respectively (Mireau *et al.*, 2003). This construct was inserted into  $\rho^+$  mtDNA, 295 bp upstream of the *COX2* gene. The endogenous *COX1* gene was replaced with *cox1Δ::ARG8<sup>m</sup>* (Materials and methods). Open boxes indicate *COX1*, *COX2* and *ARG8<sup>m</sup>* structural genes. The *COX1* and *COX2* 5'-UTLs and 3'-UTRs are indicated as thick lines. The arrows indicate the direction of transcription for each gene. **(B)** Growth phenotypes of strains carrying the ectopic chimeric *COX1* gene. Relevant nuclear genotypes are as indicated. Cells grown on liquid YPD were spotted on glucose minimal medium containing (+Arg) or lacking (–Arg) arginine and on non-fermentable ethanol/glycerol medium (YPEG), and incubated for 2 days at 30°C. The *pet111Δ* mutation removes the *COX2* mRNA-specific translational activator (Poutre and Fox, 1987; Mulero and Fox, 1993). **(C)** Pulse-labeling of mitochondrial translation products. Cells were labeled with [<sup>35</sup>S]methionine for 30 min in the presence of cycloheximide, and proteins were analyzed as described in Materials and methods. Cytochrome *c* oxidase subunit 1, Cox1; subunit 2, Cox2; subunit 3, Cox3; cytochrome *b*, Cytb; subunit 6 of ATPase, Atp6; *ARG8<sup>m</sup>* gene product, Arg8; and the ribosomal protein, Var1. Strain details are given in the Supplementary data.

We created strains containing only this construct (pXPM42, Figure 6A) as their mtDNA by mitochondrial transformation of a  $\rho^0$ , and introduced either *mss51Δ* or *pet309Δ* nuclear mutations. These strains were mated with strains carrying wild-type  $\rho^+$  mtDNA and either *mss51Δ* or *pet309Δ* mutations (Figure 6B). The resulting diploids, containing both pXPM42  $\rho^-$  and wild-type  $\rho^+$  mtDNAs heteroplasmically, were then tested for their ability to grow on Arg selective medium containing glucose, and on

non-fermentable medium. As expected, homozygous *pet309Δ* diploid cells were both Arg<sup>+</sup> and competent for respiratory growth, albeit at less than wild-type strength (Figure 6B, upper left patch), demonstrating that this chimeric *COX1(1–512)::ARG8<sup>m</sup>* mRNA could be translated in the absence of Pet309p. In contrast, homozygous *mss51Δ* diploid cells exhibited Arg<sup>–</sup> and non-respiratory phenotypes (Figure 5B, lower right patch). The Arg<sup>–</sup> phenotype of these *mss51Δ* cells confirms that translation



**Fig. 6.** Mss51p acts through the *COX1* coding sequence to promote *COX1* translation. (A) Schematic representation of the chimeric *COX1* gene present in the plasmid pXPM42. The *COX1(1–512)::ARG8<sup>m</sup>* gene (represented as open boxes) was fused to 310 bp of the *COX2* 5'-UTL and 270 bp of the *COX2* 3'-UTR. The 54 bp from the *COX2* promoter and 75 bp from the *COX2* mRNA 3' end are represented by thick lines. The black triangle indicates the position of the pre-Arg8p targeting signal cleavage site. (B) Expression of the chimeric *COX1(1–512)::ARG8<sup>m</sup>* gene in *mss51Δ* and *pet309Δ* mutants. Synthetic  $\rho^-$  haploid strains carrying pXPM42 DNA were patched in vertical stripes on YPD. Cells containing wild-type  $\rho^+$  mitochondria were patched on YPD on horizontal stripes. Their relevant genotypes are as indicated. The stripes were cross-printed on YPD and allowed to mate overnight before being replicated to glucose minimal medium lacking arginine (–Arg) and complete non-fermentable ethanol/glycerol medium (YPEG). These plates were incubated for 5 days at 30°C to reveal the phenotype of the resulting heteroplasmic diploid strains. Strain details are given in the Supplementary data.

of this chimeric mRNA depends on the action of Mss51p upon a target or targets embedded in the *COX1* codons. This activity must be distinct from the action of Mss51p on the UTRs of the *COX1* mRNA to promote translation.

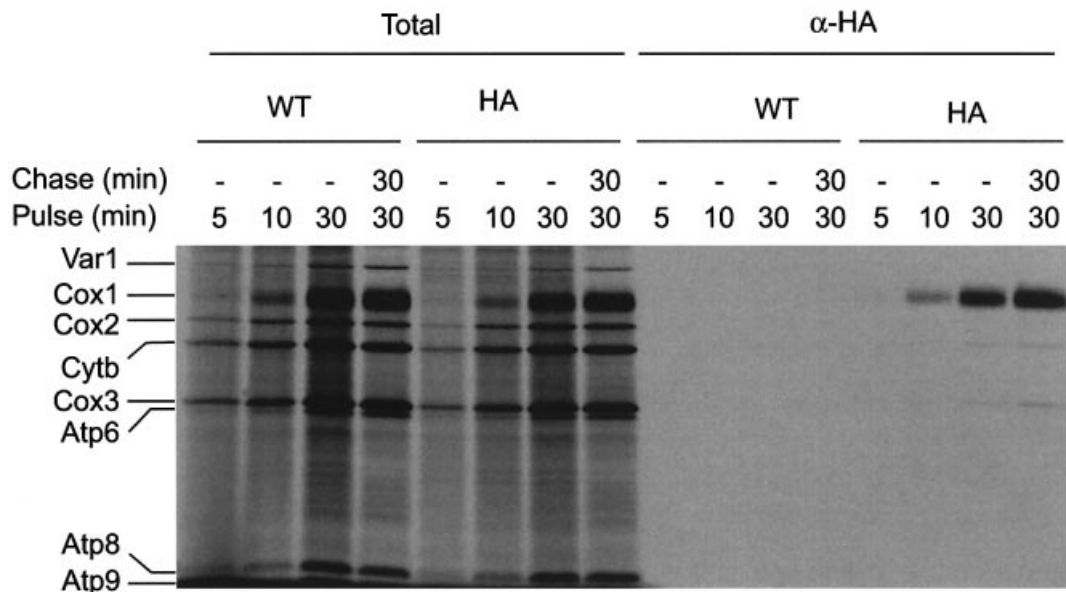
#### ***Mss51p* interacts with newly synthesized Cox1p**

We next asked whether newly synthesized Cox1p itself could be a target of Mss51p. To explore this, we attached triple hemagglutinin (HA) epitopes to the C-terminus of Mss51p for co-immune precipitation experiments. The respiratory competence of Mss51p-HA and the untagged Mss51p cells was comparable, indicating that Mss51p-HA was functional. Translation in mitochondria isolated from strains bearing either the *MSS51-HA* or the *MSS51* gene was carried out in the presence of [<sup>35</sup>S]methionine for 5–30 min, and then the mitochondria were solubilized with digitonin. Interaction with newly synthesized Cox1p was assessed by immunoprecipitation using an HA-specific antibody, and the immunoprecipitate was analyzed by SDS–PAGE. Radiolabeled Cox1p was detectable in both the total mitochondrial samples and in the immunoprecipitate from the *MSS51-HA* mitochondria after the 5 min

pulse, and increased in amount during the course of the experiment (Figure 7). The labeled Cox1p was stable during a 30 min chase, as was its interaction with Mss51p-HA. The newly synthesized Cox1p was not immunoprecipitated from mitochondria containing untagged Mss51p, demonstrating specificity of the reaction. The immunoprecipitates from both extracts contained trace amounts of cytochrome *b* and Cox3p, although these signals were slightly stronger in the presence of Mss51p-HA. Interaction of newly synthesized Cox1p with Mss51p-HA was also observed when immunoprecipitation was performed in mitochondria solubilized with Triton X-100 (data not shown).

#### **Discussion**

We have examined the role of the nuclear gene product Mss51p in translation of the mitochondrial *COX1* mRNA, and have found that it is remarkably different from those of previously described mRNA-specific translational activators. The *COX1*, *COX2* and *COX3* mitochondrially coded mRNAs are translated only when their previously



**Fig. 7.** Mss51p interacts with newly synthesized Cox1p. Translation in mitochondria isolated from *MSS51* (WT) or *MSS51-HA* (HA) strains was performed at 25°C in the presence of [<sup>35</sup>S]methionine. Samples were removed at the indicated labeling times (pulse). A fourth sample was pulse-labeled for 30 min and then chased by addition of 10 mM cold methionine followed by incubation at 25°C for 30 min (chase). Mitochondria were washed and solubilized, and the solubilized products were immunoprecipitated with anti-HA antibody (α-HA). Translation products were analyzed by SDS-PAGE and autoradiography. The total aliquots represent 10% of the aliquots used for immunoprecipitation. Abbreviations are as in Figure 5C; ATPase subunit 8, Atp8; subunit 9, Atp9. Strain details are given in the Supplementary data.

described mRNA-specific activators are present and can functionally interact with the target mRNA 5'-UTLs (Fox, 1996). Interaction of these activators with each other is likely to promote correct localization of translation on the inner membrane (Sanchirico *et al.*, 1998; Naithani *et al.*, 2003). The requirement for any one of these three translational activators can be bypassed by replacing its target 5'-UTL with the 5'-UTL of an mRNA encoding another mitochondrial membrane protein (Costanzo and Fox, 1986; Mulero and Fox, 1993; Manthey and McEwen, 1995). Our evidence shows that nuclear-encoded Mss51p has a novel role in controlling the mitochondrial synthesis of Cox1p. Unlike mutations affecting other mRNA-specific translational activators, including the *COX1* mRNA-specific activator Pet309p, *mss51* mutations have never been suppressed by mitochondrial gene rearrangements that allow other translational activators to work on the *COX1* mRNA (Decoster *et al.*, 1990) (our unpublished results). Here, we have confirmed that a chimeric *COX3-COX1* mRNA that is translated in the absence of Pet309p (Manthey, 1995; Manthey and McEwen, 1995) cannot be expressed in the absence of Mss51p. Thus the role of Mss51p in allowing Cox1p accumulation is mechanistically distinct from that of Pet309p and the other mRNA-specific translational activators described to date. Indeed, it seemed possible that the apparent lack of Cox1p synthesis in *mss51* mutants could be due to extremely rapid degradation of pulse-labeled protein.

To re-examine the role of Mss51p in Cox1p accumulation, we employed the synthetic mitochondrial reporter gene *ARG8<sup>m</sup>* (Steele *et al.*, 1996). Since *ARG8<sup>m</sup>* expression was not blocked by *mss51* mutations when it was inserted into the *COX2* and *COX3* loci, it is clear that neither its coding sequence nor its protein product, Arg8p, is

intrinsically affected by Mss51p. *ARG8<sup>m</sup>* expression depends upon *MSS51* function when *ARG8<sup>m</sup>* is substituted for the *COX1* coding sequence at the *COX1* locus. This clearly demonstrates that *MSS51* is required for translation of the reporter mRNA bearing *COX1* 5'- and 3'-UTRs in the absence of *COX1* codons. *ARG8<sup>m</sup>* expression was also *MSS51* dependent when the reporter sequence was fused to the end of the *COX1* coding sequence. These observations, taken together with the inability of a 5'-UTL substitution to bypass the requirement for *MSS51*, demonstrate that Mss51p has a specific yet novel role in promoting translation of the *COX1* mRNA, that must involve at least one target outside the mRNA 5'-UTL. Our results cannot distinguish possible roles for the *COX1* 5'-UTL, 3'-UTR or both as the targets of Mss51p action, nor whether it acts directly on the mRNA.

Surprisingly, in addition to being required for translation of mRNAs bearing *COX1* mRNA UTRs, Mss51p was also required for translation of chimeric mRNAs bearing the *COX1* coding sequence, even when no *COX1* untranslated sequences were present. We inserted into an ectopic site in mtDNA the *COX1* coding sequence, flanked by the *COX2* promoter and 5'-UTL, and by the *COX2* 3'-UTR. As expected, Cox1p encoded by this chimeric gene was both synthesized and functionally expressed at the level of respiratory growth in a *pet309Δ* strain, since the *COX2* mRNA-specific activator Pet111p could substitute for Pet309p. However, Cox1p encoded by this chimeric gene could not be synthesized in the absence of Mss51p. Since Mss51p is not required for expression of either *COX2* or *ARG8<sup>m</sup>* coding sequences at the *COX2* locus, these data show that Mss51p must have a target of action specified by the *COX1* coding sequence. Moreover, the requirement for Mss51p for expression of *COX1*



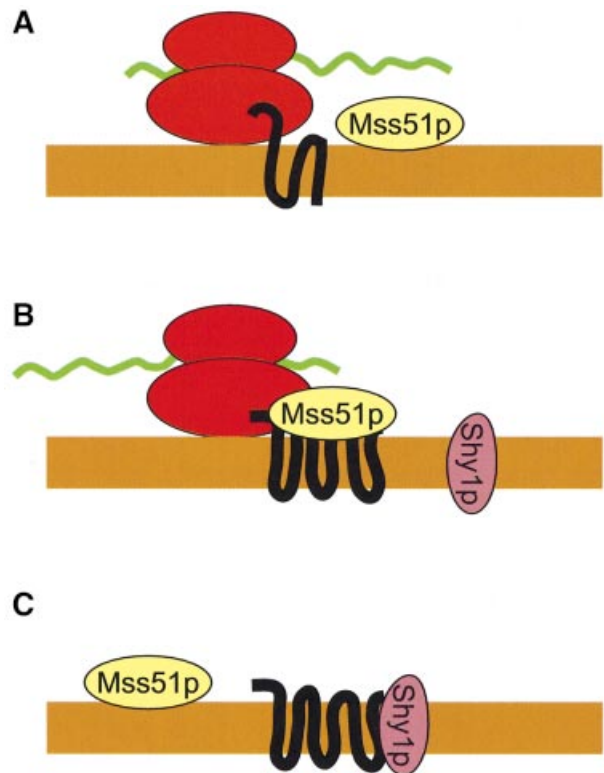
codons is at the translational level, since the expression from the ectopic *COX2* locus of the reporter *ARG8<sup>m</sup>* fused to the *COX1* coding sequence also exhibited a strong requirement for *MSS51*.

Our findings suggest that Mss51p has a role in translation elongation during Cox1p synthesis. It was observed previously that the negative effect of the *mss51-3* mutation, which lowers the levels of wild-type Mss51p, is enhanced when combined with the paromomycin resistance mutation *P<sup>R454</sup>* in mtDNA (Decoster *et al.*, 1990; Siep *et al.*, 2000). This supports a role for Mss51p during elongation since *P<sup>R454</sup>* affects a conserved region of the mitochondrial 15S rRNA (Li *et al.*, 1982), reducing the ability of paromomycin to interfere with the decoding process at the ribosomal A-site (Fourmy *et al.*, 1998).

How might Mss51p function through both the UTRs and coding sequence of the *COX1* mRNA to allow its translation? Mss51p could act together with Pet309p through the *COX1* mRNA 5'-UTL to activate translation. However, Mss51p may have an entirely different role that depends upon the *COX1* mRNA 3'-UTR or both the 5'-UTL and 3'-UTR. In any event, another target of Mss51p action maps within the *COX1* coding sequence, suggesting that it could govern translation elongation through a regulatory element within the mRNA coding sequence or nascent polypeptide.

We previously have detected antagonistic translational regulatory elements embedded in the *COX2* mRNA coding sequence (Bonney *et al.*, 2001; Williams and Fox, 2003). In that case, a positive element embedded in the mRNA sequence specifying the leader peptide of yeast pre-Cox2p is required to allow efficient translation through three downstream negative elements in the mRNA coding sequence. Mss51p could recognize analogous signals in the *COX1* mRNA coding sequence, at the level of RNA. On the other hand, we found that Mss51p interacts with newly synthesized Cox1p, and it is tempting to speculate that Cox1p itself may be the target that Mss51p recognizes to promote translation. In one possible model, the nascent Cox1p polypeptide could inhibit completion of translation by the elongating ribosome. While we have not established this, regulation of translation by nascent chains is a well established phenomenon (Lovett and Rogers, 1996; Tenson and Ehrenberg, 2002). In this model, if Mss51p is available to bind to Cox1p, then translation could proceed to completion. The binding of Mss51p to Cox1p could then promote productive interactions with Shy1p and possibly other assembly proteins (Figure 8). Release of Mss51p at this stage of the assembly pathway would allow it to interact with another nascent Cox1p. By mechanisms such as this, Mss51p could tightly coordinate Cox1p synthesis with downstream events leading to cytochrome oxidase assembly.

Regulation of translation by feedback controls from membrane insertion is an established phenomenon. Recently, evidence has been presented that completion of *Escherichia coli* *secM* translation elongation is modulated by a nascent polypeptide sequence that interacts with the ribosome exit tunnel, blocking further translation if membrane insertion is stalled (in this case, regulating translation of the neighboring *secA* coding sequence) (Nakatogawa and Ito, 2002). Feedback translational control systems monitoring protein complex assembly have



**Fig. 8.** A model for regulation of Cox1p synthesis based on its physical interaction with Mss51p. This model is based on the possibility that the target specified by the *COX1* coding sequence, through which Mss51p promotes Cox1p synthesis, is Cox1p itself. We propose the following. (A) Translation elongation of the *COX1* mRNA (green line) may be arrested by interaction of the nascent Cox1p polypeptide (black, thick line) with the ribosome. (B) Interaction of Mss51p with nascent Cox1p allows completed translation. (C) Mss51p 'hands off' newly synthesized Cox1p to Shy1p, and possibly other proteins, for assembly. Mss51p is then released and available to interact with another Cox1p nascent polypeptide as shown in (A). The mitochondrial inner membrane is shown as a brown bar. For simplicity, the action of Mss51p through the *COX1* mRNA UTRs to promote translation was not included in this model, since the target could involve the *COX1* 5'-UTR, the 3'-UTR or both.

also been described for flagella in bacteria (Aldridge and Hughes, 2002) and for photosystem complexes in chloroplasts (Choquet *et al.*, 2001). One can easily imagine that the presence of unassembled Cox1p in the mitochondrial inner membrane could be deleterious, and that mechanisms to prevent its synthesis in the absence of assembly may have evolved.

Mitochondrial genetic systems have diverged dramatically during their evolution from an apparent common ancestor (Gray *et al.*, 1999). The extent to which common regulatory mechanisms may have been conserved remains unclear. The *SURF1/SHY1* cytochrome oxidase assembly function appears to be conserved from yeast to humans. Proteins highly similar to *S.cerevisiae* Mss51p, and likely to be orthologous, are encoded in the genomes of both *Neurospora crassa* and *Schizosaccharomyces pombe* (accession Nos EAA28045 and T50191, respectively), as well as closely related budding yeasts (Cliften *et al.*, 2003; Kellis *et al.*, 2003). No clear homologs of Mss51p are present in currently available animal genomes; however, highly diverged orthologs may be present in animals.

## Materials and methods

### Strains, media and genetic methods

*Saccharomyces cerevisiae* strains used in this study are listed in Supplementary table IS. Standard genetic methods and media recipes were as previously described (Rose *et al.*, 1988; Fox *et al.*, 1991). Complete fermentable media were YPD or YPRaf (containing 2% glucose or 2% raffinose). Non-fermentable medium was YPEG (3% glycerol, 3% ethanol) or 2% lactate (Diekert *et al.*, 2001). Minimal medium was synthetic complete (0.67% yeast nitrogen base, 2% glucose) lacking arginine. The *mss51Δ::LEU2* and *shy1Δ::URA3* deletion constructs were obtained by PCR. The *pet309::URA3* disruption cassette was obtained from Manthey and McEwen (1995). *Mss51p* was tagged at its C-terminus with an HA<sub>3</sub> epitope by transformation of DAU1 with a PCR product (Schneider *et al.*, 1995).

Plasmids pXPM18, pXPM19, pXPM34i and pXPM42 (see below) were transformed into the  $\rho^0$  strain NAB69 by high-velocity microprojectile bombardment (Bonney and Fox, 2001). Transformants were selected by their ability to rescue arginine growth when mated with an *arg8<sup>m</sup>-1* mutant (Bonney and Fox, 2000), or by the ability of the resulting diploids to respire when mated with a strain carrying a *cox1D369N* mutation, L45 (Meunier *et al.*, 1993).

Transformants with plasmids pXPM18 and pXPM19 (see below) were mated with XPM46 (containing intronless mtDNA; Labouesse, 1990) or NB40-36a (intron-bearing mtDNA derived from D273-10B mitochondria). Cytoductants were selected for their ability to grow on media lacking arginine. The mitochondrial transformant of plasmid pXPM34i (see below) was mated by cytoduction with XPM13a, which carries the *cox2-62* mutation (Dunstan *et al.*, 1997) and the *cox1Δ::ARG8<sup>m</sup>* construct, and respiring recombinants were selected. In all cases, correct integration of the different constructs into the mtDNA was confirmed by PCR and DNA sequencing.

### Construction of chimeric mitochondrial genes

All plasmids were generated by the fusion PCR technique (Ho *et al.*, 1989) using *Pfu* polymerase (Stratagene) or *Taq* polymerase (Invitrogen). Total DNA from the strain NB40-36a or plasmid pHd6 (Green-Willms *et al.*, 2001) was used to amplify *COX1* and *ARG8<sup>m</sup>* sequences, respectively. For the *COX1(1-512)::ARG8<sup>m</sup>* construct, the *ARG8<sup>m</sup>* codons specifying the precursor Arg8p were fused in-frame to the last exon (453 bp) of the *COX1* coding sequence, and upstream 986 bp of the *COX1* 3'-UTR. This product was ligated into the *PstI-XhoI* sites of pBluescript to generate plasmid pXPM18. This plasmid was used to integrate the *COX1(1-512)::ARG8<sup>m</sup>* construct into the intronic *COX1* locus derived from D273-10B mitochondria or the intronless *COX1* locus derived from CK520 mitochondria (Labouesse, 1990). The *cox1Δ::ARG8<sup>m</sup>* construct was obtained as a precise fusion of the *ARG8<sup>m</sup>* gene specifying the precursor Arg8p downstream 790 bp of the *COX1* 5'-UTL and upstream 986 bp from the *COX1* 3'-UTR to create plasmid pXPM19 (Figure 1).

Intronless mtDNA of strain XPM46, derived from CK520, and the plasmid pJM2 (Mulero and Fox, 1993) were used as templates for PCRs to amplify *COX1* or *COX2* sequences, respectively. Using the QuikChange mutagenesis kit (Statagene), a *SnaBI* site was introduced in pJM2 at position -295 relative to the *COX2* start codon to create plasmid pXPM50. The chimeric *COX1* gene, with 73 and 119 bp of the *COX2* 5'-UTL and 3'-UTR (Mireau *et al.*, 2003), respectively, was amplified by fusion PCR. The primers used introduced *EcoRV* sites at the ends of the PCR product. This amplification product was digested with *EcoRV* and ligated into the *SnaBI* site of pXPM50 to create plasmid pXPM34i. In this construct, the *COX1* chimeric gene was integrated in the opposite orientation to *COX2* (Figure 5).

Plasmid pXPM42 was obtained by a precise fusion of the *COX1(1-512)::ARG8<sup>m</sup>* codons to 310 and 271 bp of the *COX2* 5'-UTL and 3'-UTR, respectively (Figure 7). DNA from the strain XPM78a or from pJM2 was used to amplify *COX1(1-512)::ARG8<sup>m</sup>* or *COX2* sequences respectively.

### Analysis of mitochondrial proteins

Yeast cells were grown in complete raffinose medium until late log phase. Cells were disrupted by vortexing with glass beads, and crude mitochondria were obtained as described by Diekert *et al.* (2001), except that protease inhibitor cocktail (Sigma) was added instead of phenylmethylsulfonyl fluoride (PMSF). Proteins were separated by SDS-PAGE on a 12.5% gel (Laemmli, 1970), and western blots were probed with an anti-Arg8p antibody (Steele *et al.*, 1996) or an anti-citrate synthase antibody (provided by G.Schatz). Secondary goat anti-rabbit

IgG conjugated to horseradish peroxidase (Bio-Rad) was detected with the ECL kit (Amersham Pharmacia Biotech).

*In vivo* pulse-labeling of cells with [<sup>35</sup>S]methionine was performed as previously described (Bonney *et al.*, 2001). After pulse-labeling, cells were chilled on ice in the presence of 10 mM cold methionine. Cells were disrupted by vortexing with glass beads and mitochondria were prepared according to Diekert *et al.* (2001). The radiolabeled proteins were separated on a 15% polyacrylamide gel and analyzed with a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Translation in isolated mitochondria (3 mg protein/ml) from *MSS51-HA* or *MSS51* strains in the presence of [<sup>35</sup>S]methionine was performed as previously described (Westermann *et al.*, 2001). After translation, mitochondria were washed with 0.6 M sorbitol, 20 mM HEPES pH 7.4, and lysed with a buffer containing 1% digitonin, 100 mM NaCl and 20 mM Tris pH 7.4. Immunoprecipitation of labeled mitochondrial products with an anti-HA antibody (clone 3F10, Roche) was performed as previously described (Herrmann *et al.*, 2001).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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