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Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*

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Mutations in *SURF1*, the human homologue of yeast *SHY1*, are responsible for Leigh's syndrome, a neuropathy associated with cytochrome oxidase (COX) deficiency. Previous studies of the yeast model of this disease showed that mutant forms of Mss51p, a translational activator of *COX1* mRNA, partially rescue the COX deficiency of *shy1* mutants by restoring normal synthesis of the mitochondrially encoded Cox1p subunit of COX. Here we present evidence showing that Cox1p synthesis is reduced in most COX mutants but is restored to that of wild type by the same *mss51* mutation that suppresses *shy1* mutants. An important exception is a null mutation in *COX14*, which by itself or in combination with other COX mutations does not affect Cox1p synthesis. Cox14p and Mss51p are shown to interact with newly synthesized Cox1p and with each other. We propose that the interaction of Mss51p and Cox14p with Cox1p to form a transient Cox14p–Cox1p–Mss51p complex functions to downregulate Cox1p synthesis. The release of Mss51p from the complex occurs at a downstream step in the assembly pathway, probably catalyzed by Shy1p.

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Introduction

Assembly of yeast cytochrome oxidase (COX) requires the assistance of at least 20 nuclear gene products (McEwen *et al*, 1986; Tzagoloff and Dieckmann, 1990). These proteins act at all stages of the assembly process, including processing (Seraphin *et al*, 1989) and translation (Costanzo and Fox, 1993) of the mitochondrially encoded mRNAs for subunits 1, 2, and 3, membrane insertion of the hydrophobic subunits (Hell *et al*, 2001), and maturation of the heme and copper centers (Glerum *et al*, 1996a, b; Barros *et al*, 2001).

Although much progress has been made in understanding some of the events leading to the assembly of COX, the functions of a number of gene products essential for this process have yet to be clarified (Barrientos *et al*, 2002a, b). A case in point is Shy1p, a yeast mitochondrial protein needed for full expression of COX (Mashkevich *et al*, 1997; Barrientos *et al*, 2002b). The function of this protein is of considerable interest because mutations in its human homologue, Surf1p, have been shown to be responsible for most diagnosed cases of Leigh's syndrome (Tiranti *et al*, 1998; Zhu *et al*, 1998), a neuromuscular disease presenting a COX deficiency (DiMauro and De Vivo, 1996).

Extragenic suppressors of *shy1* null mutants have been mapped to *MSS51*, a nuclear gene coding for a Cox1p-specific translational activator (Barrientos *et al*, 2002b), suggesting that unassembled Cox1p may downregulate its own translation by competitively trapping a translational activator complex in which Mss51p is one of the components. According to this model, the translational block is relieved by Shy1p-dependent assembly of Cox1p making Mss51p available for Cox1p synthesis (Barrientos *et al*, 2002b).

In the present study, we show that Cox1p synthesis is reduced in most COX assembly mutants, *cox14* mutants being an exception. Mutant forms of Mss51p are able to restore Cox1p expression in strains carrying null alleles of either COX structural genes or genes coding for COX assembly factors. We also present evidence for an interaction of newly synthesized Cox1p with Mss51p and Cox14p, but not Shy1p. We propose that the formation and turnover of Cox1p/Mss51p/Cox14p couple Mss51p-dependent translation of Cox1p to its utilization for COX assembly.

Results

Synthesis of Cox1p is repressed in assembly-arrested mutants

To ascertain if the Cox1p labeling defect previously noted in *shy1* mutants (Barrientos *et al*, 2002b) is general to all mutants blocked in COX assembly, a wide range of strains with lesions in COX subunits or assembly factors were pulsed with [^{35}S]methionine *in vivo* in the presence of cycloheximide. Labeling of Cox1p, but not Cox2p or Cox3p, was visibly reduced in most mutants (Figures 1–3). The only exception was the *cox14* mutant (Glerum *et al*, 1995). The precise function of Cox14p is not known at present.

The reduced Cox1p labeling in the mutants was seen even with 5 min pulses, the shortest time at which incorporation of [^{35}S]methionine into the mitochondrial translation products could be consistently detected in the wild type. This phenotype is easy to explain in the case of the *mss51* and *pet309* mutants, both of which have mutations in Cox1p-specific translational factors (Decoster *et al*, 1990; Manthey and McEwen, 1995). Similarly, the poor labeling of Cox1p in the *oxa1* mutant (Figure 3) may be the result of rapid turnover when Oxa1p-dependent membrane insertion of Cox1p is blocked (Hell *et al*, 2001). The Cox1p deficit in the other

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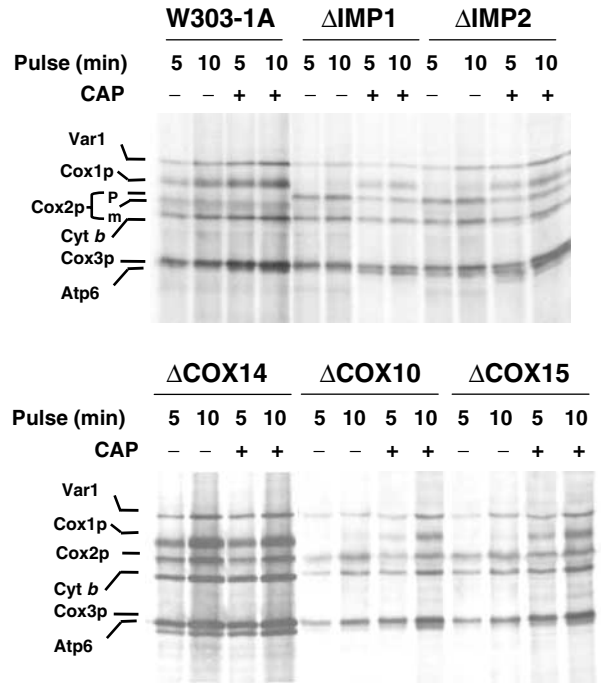

Figure 1 *In vivo* labeling of mitochondrial gene products in COX mutants. Wild-type (W303-1A) and mutant cells (described in Table I) were labeled with [^{35}S]methionine at 30°C for the indicated times in the presence of cycloheximide. One-half of each culture was incubated in the presence of 2 mg/ml chloramphenicol during the last 2 h of growth prior to labeling (+CAP). Samples were removed after the indicated times of labeling and processed as detailed in Materials and methods. The mitochondrial translation products are identified in the margin. Cox2p is not processed in ΔIMP1 and ΔIMP2 mutants. The Cox2p precursor (p) in these strains migrates slower that the mature Cox2p (m).

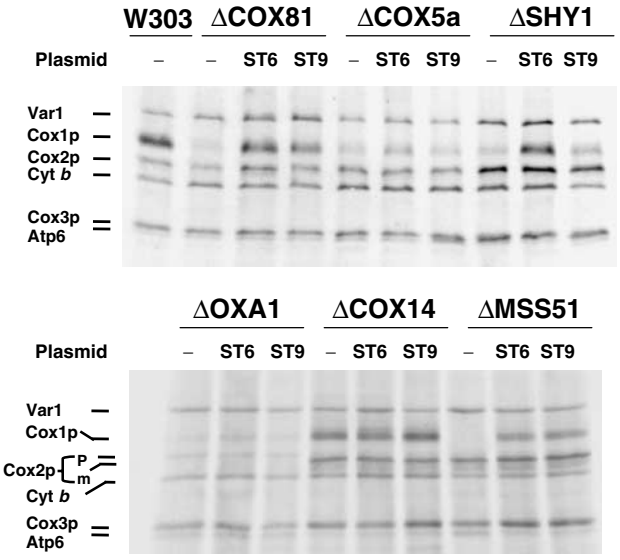

Figure 2 *In vivo* labeling of mitochondrial gene products in COX mutants expressing different alleles of *MSS51*. *MSS51* and the suppressor *mss51*^{T167R}, which partially suppress the respiratory defect of *shy1* mutants, were cloned in YIp351. The resultant constructs pSG91/ST9 and pSG91/ST6, respectively, were integrated at the chromosomal *LEU2* locus of the indicated mutants (see Table I for description of mutants). The mutants (–) and transformants were labeled with [^{35}S]methionine at 30°C for 15 min in the presence of cycloheximide.

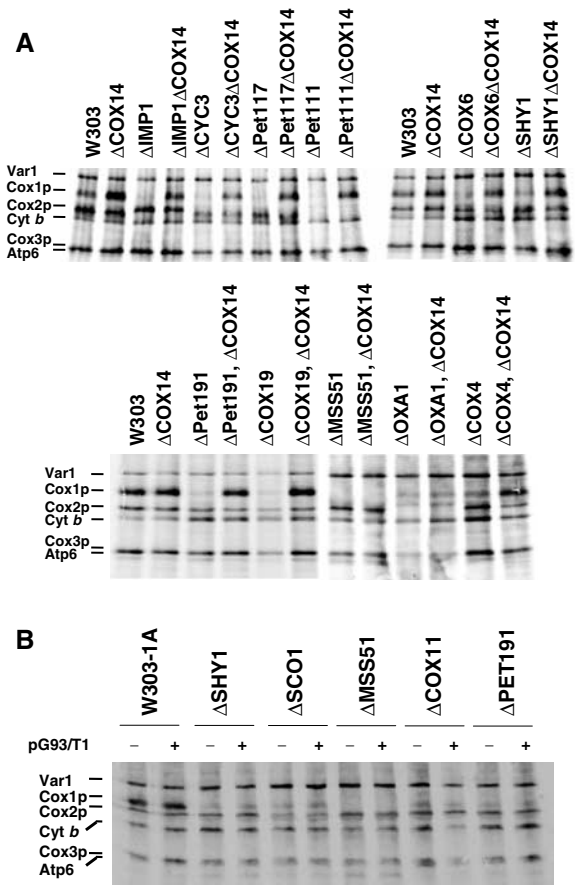

Figure 3 *In vivo* labeling of mitochondrial gene products in *COX14* mutants and effect of overexpression of COX14p on Cox1p labeling. (**A**) Wild type (W303), different COX mutants, and the same mutants carrying an additional null mutation in *COX14* were labeled with [^{35}S]methionine at 30°C for 15 min in the presence of cycloheximide. With the exception of *CYC3*, which codes for a cytochrome *c*-specific heme lyase, the functions affected in the different strains are described in Table I. (**B**) The wild-type W303-1A, the indicated COX mutants, and the same strains harboring *COX14* on a multicopy plasmid (pG93/T1) were pulse-labeled with [^{35}S]methionine and equivalent amounts of protein were separated by SDS–PAGE on a 17.5% polyacrylamide gel.

strains, however, is difficult to rationalize since the functions affected are unrelated to translation of this protein.

With the exception of the *mss51, pet309*, and *oxa1* mutants, Cox1p labeling was increased in mutants and in wild type when cells were preincubated in chloramphenicol prior to the pulse (Figure 1A and B). The improved translation of mitochondrial products following chloramphenicol treatment is presumed to occur as a result of the larger pools of nuclear-encoded subunits available for assembly of intermediates and/or because of the accumulation of nuclear-encoded factors required for mitochondrial gene expression. Methionine incorporation into Cox1p in the mutants after the chloramphenicol incubation was comparable to that seen in wild type under normal pulse-labeling conditions, indicating that the translation apparatus is fully functional in the mutants and that the phenotype stems from a decreased rate of synthesis and/or increased rate of turnover of Cox1p. Turnover seemed less likely in view of the ability of *cox14* mutants to synthesize Cox1p at rates similar to wild type

even though they are also blocked in COX assembly and displays low steady-state concentrations of Cox1p and Cox2p (Glerum *et al*, 1995). This and other observations discussed below favor decreased translation as the more likely explanation for the observed deficit of newly synthesized Cox1p in the various COX-deficient mutants.

Cox1p synthesis defect in COX assembly mutants in suppressed by the mss51^{T167R} allele

A single copy of the *mss51*^{T167R} allele or an extra copy of wild-type *MSS51* was shown to suppress partially the respiratory defect of *shy1* mutants by increasing Cox1p translation (Barrientos *et al*, 2002b). This suggested that the *shy1* mutation may inactivate or reduce the effective concentration of Mss51p as a translational activator of the *COX1* mRNA. These observations have been extended to other COX-deficient mutants. Mitochondrial translation products were labeled *in vivo* with [^{35}S]methionine in an assortment of COX mutants, with and without an extra copy of the wild-type *MSS51* or the *mss51*^{T167R} suppressor integrated at the *leu2* locus of nuclear DNA. In all the strains except the *cox14* mutant examined, synthesis of Cox1p, but not of the other COX subunits, was markedly increased by the suppressor and to a lesser extent also by the extra copy of wild-type *MSS51* (Figure 2).

It is significant that the higher expression of Cox1p leads to a partial rescue of respiration and COX activity in the *shy1* mutant (Barrientos *et al*, 2002b), but not in any of the other mutants examined. This suggests that the functions of Shy1p and Mss51p are related. In other COX mutants, however, restoration of normal rates of Cox1p synthesis by the *mss51*^{T167R} suppressor (see ΔCOX18 in Figure 2) is not a sufficient condition to compensate for the assembly defect because the impaired function is unrelated to expression of this subunit.

Increased Cox1p synthesis in cox14 mutants is epistatic in mutants with lesions in other COX assembly factors

The lack of effect of the *cox14* mutation on Cox1p synthesis (Figures 1–4) suggested that Cox14p might negatively regulate translation of this subunit. This was tested by measuring Cox1p synthesis in strains carrying mutations in *COX14* and other COX-specific genes. The *cox14* mutation restored normal Cox1p synthesis in all the COX mutants except the *mss51*, *pet309* (not shown), and *oxa1* mutants (Figure 3A). Despite

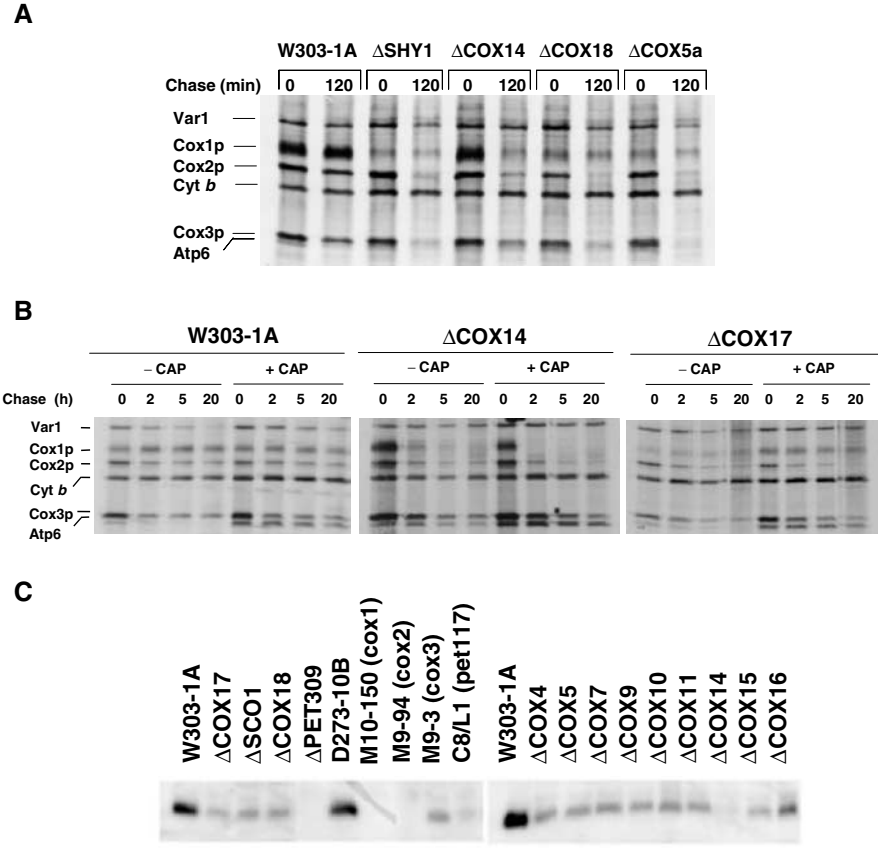

Figure 4 Turnover of *in vivo*-labeled mitochondrial translation products and steady-state concentration of Cox1p in wild type and COX mutants. (**A**) Wild type (W303-1A) and mutants (described in Table I) were grown and labeled for 20 min at 30°C with [^{35}S]methionine. Labeling was terminated by addition of 80 μmol 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. (**B**) The wild-type strain W303-1A and the cox*14* and *cox17* mutants were labeled and chased for the indicated times as in panel A. One-half of each culture was incubated in the presence of chloramphenicol as in Figure 1 prior to labeling. (**C**) The wild-type W303-1A and D273-10B and mutant strains were grown in 2% galactose, 1% yeast extract, and 2% peptone to stationary phase. Mitochondria were prepared and 10 μg of protein was separated by SDS–PAGE on a 12% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with a polyclonal antibody against yeast Cox1p. The antibody–antigen complexes were visualized by a secondary reaction with [^{125}I]protein A.

being epistatic with respect to Cox1p expression, the *cox14* mutation did not rescue either respiration or the ability of the mutants, including the of *shy1* mutant, to assemble COX (not shown).

Overexpression of Cox14p does not alter synthesis of Cox1p

Normal labeling of Cox1p in *cox14* single and double mutants could indicate that Cox14p acts to increase degradation of unassembled or incompletely assembled Cox1p. If this were the case, overexpression of Cox14p in a wild-type or mutant background might be expected to affect the amount of newly synthesized Cox1p. This was examined by transforming the wild-type strain and several COX assembly-deficient mutants with the episomal plasmid pG93/T1 (Glerum *et al*, 1995), which contains a wild-type *COX14* gene. Overexpression of Cox14p in these strains did not affect *in vivo* labeling of Cox1p (Figure 3B) or restore respiration (data not shown).

Stability of newly synthesized Cox1p in wild type and COX mutants

The stability of unassembled Cox1p was assessed in wild type and different COX mutants by pulse-chase. Most of the translation products, including the three COX subunits, were stable during 2 h of chase in wild type but not in the mutants in which a significant fraction of Cox2p and Cox3p were degraded (Figure 4A). In contrast, the small amount of labeled Cox1p detected in the mutants was stable during the chase (Figure 4A). The exception was the *cox14* mutants, in which most of the Cox1p was degraded (Figure 4A and B). The kinetics of Cox1p turnover was also examined during longer periods of chase of cells pulse-labeled with and without a prior incubation in the presence of chloramphenicol (Figure 4B). Cox1p was stable even after 20 h of chase, but under the same conditions most of Cox1p in the *cox14* mutant was degraded after 2 h of chase. The small amount of Cox1p detected in the *cox17* mutant appears to be as stable as in wild type. The greater lability of Cox1p in the *cox14* mutant is also supported by the results of Western analysis of the steady-state concentrations of this subunit in different mutants (Figure 4C).

Cox1p, Cox2p, and Cox3p synthesized in cycloheximide-inhibited wild-type cells are not incorporated into the holoenzyme, even following a 2 h period of chase (data not shown). The relatively high stability of these COX subunits, however, suggests that they are in a protease-protected environment either as monomers or partially assembled intermediates, or that they are complexed to a 'stabilizing' factor(s).

Do Shy1p and Cox14p act post-translationally?

Manthey and McEwen (1995) have shown that ρ^- genomes in which *COX1* is fused to the 5′ leader of the mitochondrial *COB* (SUP2; see Figure 5A) or C*OX3* (SUP1) genes are able to suppress *pet309* mutants. This argues strongly against a post-translational role of Pet309p in expression of COX. In this study, we have constructed strains heteroplasmic for wild type and the SUP2 ρ^- suppressor in the context of *shy1*, *mss51*, or *cox14* null mutations.

The failure of the SUP2 suppressor to rescue *cox14* and *shy1* mutants (Figure 5B) indicates that Cox14p and Shy1p are required at a post-translational stage of COX assembly,

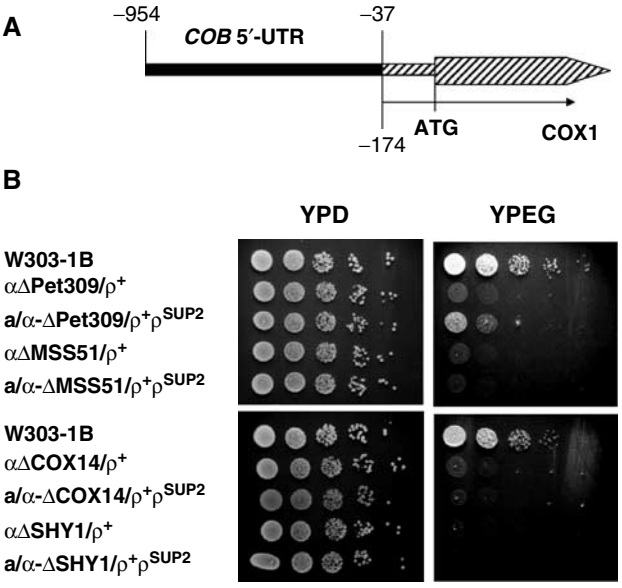

Figure 5 The *cox14* defect is not rescued by a ρ^- genome with *COX1* fused to the 5′-UTR of *COB*. (**A**) Map of the mitochondrial bypass suppressor ρ^{SUP2}. The rearrangement in the suppressor leads to a fusion of the 5′-UTR of *COB* to nucleotide −174 of *COX1* (Manthey and McEwen, 1995). (**B**) ρ^{SUP2} was transferred by cytoduction to a *kar1* mutant (Conde and Fink, 1976) lacking mitochondrial DNA (ρ°). The SUP2 suppressor was transferred from the *kar1* donor to ρ° derivatives of null mutants of *PET309*, *MSS51*, *COX14*, and *SHY1*. The different mutants with the SUP2 genome (ρ^{SUP2}) were then crossed to the isogenic mutants with wild-type mitochondrial DNA (ρ^+) to obtain the heteroplasmic diploid mutants of *PET309* (a/α-ΔPET309/$\rho^+\rho^{SUP2}$), *MSS51* (a/α-ΔMSS51/$\rho^+\rho^{SUP2}$), *COX14* (a/α-ΔCOX14309/$\rho^+\rho^{SUP2}$), and *SHY1* (a/α-ΔSHY1/$\rho^+\rho^{SUP2}$). Serial dilutions of the haploid mutants with wild-type mitochondrial DNA and of the diploid strains with the wild-type and suppressor genomes were spotted on YPD and YPEG plates and incubated at 30°C for 2.5 days.

although it does not exclude a role in translation as well. In agreement with the findings of Perez-Martinez *et al* (2003) who used SUP1 in their studies, SUP2 does not restore the COX deficiency of *mss51* mutants (Figure 5B).

Cox14p interacts with newly synthesized Cox1p

Restoration of normal Cox1p translation in mutants that have a second mutation in *COX14* suggested that Cox14p might be regulating Cox1p synthesis. A physical interaction of Cox1p with Cox14p was tested by expressing the latter as a GST fusion protein from a chromosomally integrated gene. The GST-tagged Cox14p was able to fully complement the respiratory defect of the *cox14* mutant (data not shown). Mitochondria from aW303ΔCOX14/ST32 expressing the Cox14p-GST fusion protein were labeled *in organello* with [^{35}S]methionine, extracted with lauryl maltoside, and adsorbed onto glutathione–Sepharose beads. The proteins that were recovered from the beads indicated a selective and virtually quantitative enrichment of labeled Cox1p (Figure 6A). A similar enrichment of Cox1p was seen when mitochondria were isolated from a strain expressing Mss51p-GST but not from wild type or from a strain expressing a Shy1p-GST fusion protein. Like Cox14p-GST, the latter two fusions also complemented the respective null mutants. An association of HA-tagged Mss51p with newly synthesized Cox1p was also reported by Perez-Martinez *et al* (2003).

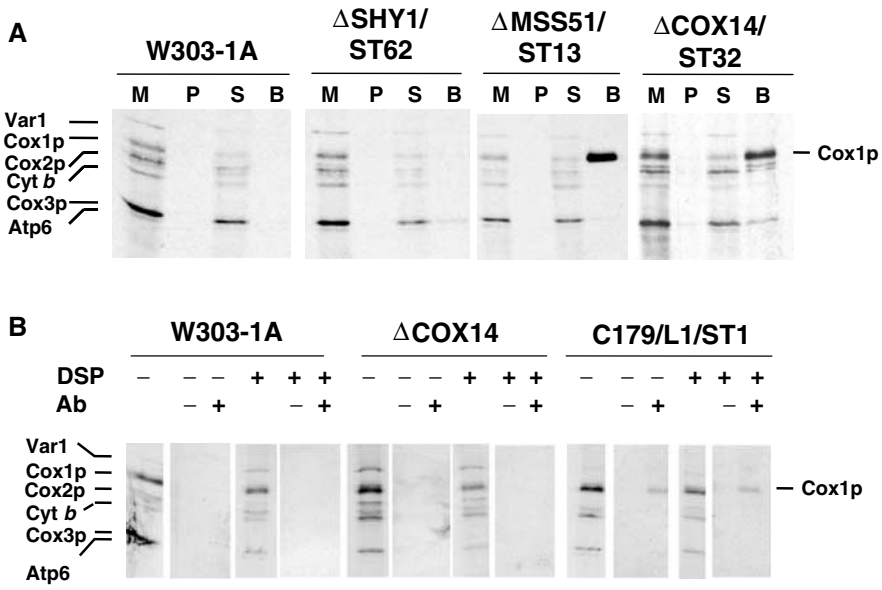

Figure 6 Cox14p and Mss51p interact with Cox1p. (**A**) Mitochondria were prepared from the wild-type W303-1A, a *shy1* null mutant (ΔSHY1/ST62) with a chromosomally integrated plasmid expressing the Shy1p-GST fusion protein, an *mss51* null mutant (ΔMSS51/ST13) with a chromosomally integrated plasmid expressing Mss51p-GST, and a *cox14* null mutant (ΔCOX14/ST32) with a chromosomally integrated plasmid expressing Cox14p-GST. Mitochondria were labeled with [^{35}S]methionine for 30 min and extracted with 1% lauryl maltoside, 1 M KCl, and 1 mM PMSF. 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 supernatant was collected and the beads were washed three times with PBS. Mitochondria (M) corresponding to 2 μg protein, equivalent volumes of the membrane pellet (P) after lauryl maltoside extraction and of the supernatant from the glutathione–Sepharose beads (S) were separated on a 17.5% polyacrylamide gel by SDS–PAGE. The amount of washed beads (B), however, corresponded to ~500 μg of the starting mitochondria. (**B**) Mitochondria from W303-1A, the *cox14* null mutant (ΔCOX14), and a *cox14* point mutant transformed with a high-copy plasmid containing *COX14* (C179/L1/ST1) were labeled for 30 min at 30°C in the presence of [^{35}S]methionine. After a 5 min pulse, the samples were treated with the crosslinker DSP (+) or were mock-treated (−) as described (Hell *et al*, 2000). Immunoprecipitation of crosslinked adducts was performed using antiserum specific for Cox14p (+) and preimmune serum (−). Immunoprecipitates were analyzed by SDS–PAGE and autoradiography as in Figure 1.

Trace amounts of cytochrome *b*, Cox2p, and Cox3p were also adsorbed to the beads but the signals varied in different experiments. The enrichment of the Cox2p precursor seen in the pull-down of the strain expressing Cox14p-GST was consistent but was investigated further.

To test if the binding of Mss51p to newly synthesized Cox1p requires the presence of Cox14p, an *mss51* and *cox14* double mutant was transformed with an integrative plasmid expressing Mss51p-GST. Co-precipitation of newly synthesized Cox1p with Mss51p-GST in the *cox14* null background (data not shown) indicates that the interaction of Mss51p and Cox1p is not Cox14p dependent.

The interaction of Cox14p with newly synthesized Cox1p was also studied by labeling mitochondria *in organello* with [^{35}S]methionine in the absence or presence of the cleavable crosslinker dithio-bis-succinimidyl propionate (DSP) to trap transient complexes that might be formed early after completion of Cox1p synthesis. Detergent extracts containing the labeled translation products were treated with antibody to Cox14p and analyzed by SDS–PAGE under conditions causing cleavage of the crosslinker. A small fraction of newly synthesized Cox1p was present in the immunoprecipitate obtained with the Cox14p antibody in a strain overexpressing Cox14p but not in a wild-type strain (Figure 6B). The co-immunoprecipitation of Cox1p did not depend on the inclusion of DSP during translation. The poor recovery of Cox1p in this procedure is probably due to the low efficiency of immunoprecipitation with the Cox14p antibody.

Cox14p interacts with Mss51p

The interaction of Cox14p with Mss51p was examined in strains of yeast, expressing either Cox14p-GST or Mss51p-GST in *cox14* and *mss51* null backgrounds, respectively. Pull-down assays of mitochondria extracted with lauryl maltoside indicated that approximately 61% of Mss51p-GST and 48% of Cox14p were adsorbed onto the beads (Figure 7A). Likewise, when crude mitochondrial extracts containing Cox14p-GST were adsorbed onto glutathione–Sepharose beads, more than 75% of Cox14p-GST and 50% of Mss51p were pulled down (Figure 7B).

Part of the lauryl maltoside extracts was centrifuged on a linear 7.5–25% sucrose gradient. Gradient fractions were analyzed for the distributions of Mss51p and Cox14p and the peak fractions with Mss51p-GST and Cox14p or Cox14p-GST and Mss51p were treated with glutathione–Sepharose beads. These pull-down assays confirmed that the Cox14p cosedimenting with Mss51p-GST and vice versa were complexed to each other (data not shown). Our data, however, do not discriminate between a direct interaction of the two proteins and an interaction mediated by other proteins that may constitute the complex.

The GST pull-down assays suggested that only a fraction of Mss51p is complexed to Cox14p. This was supported by the results of sucrose gradient sedimentations of mitochondrial detergent extracts. Mss51p and Cox14p sedimented similarly in a gradient loaded with a lauryl maltoside extract of wild-type mitochondria. Both proteins peaked only a fraction

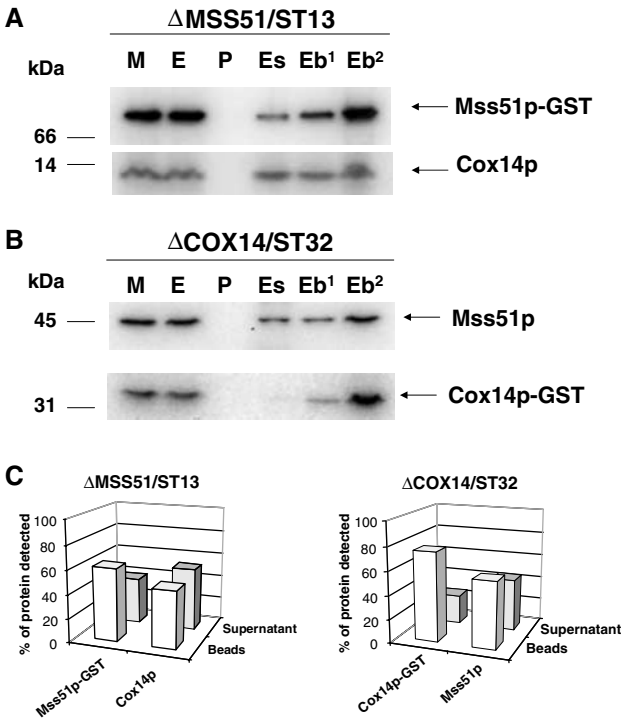

Figure 7 Cox14p interacts with Mss51p. (**A**) Mitochondria (M) from an *mss51* null mutant with a chromosomally integrated plasmid expressing Mss51p-GST fusion protein (ΔMSS51/ST13) were extracted with 1% lauryl maltoside, 1 M KCl, and 1 mM PMSF. The pellet (P) after centrifugation at 50 000 g_{av} for 30 min was suspended in the starting volume of buffer and the extract (E) was mixed and incubated for 4 h at 4°C with glutathione–Sepharose. The supernatants (Es) from the beads were collected and the beads (Eb) were washed three times with PBS. The different fractions adjusted for volume were separated by SDS–PAGE. The lane labeled (Eb^2) was loaded with two times the amount of beads. Cox14p and Mss51p-GST were detected by Western blot analysis using specific antibodies against each protein. The proteins were visualized by a secondary reaction with [^{125}I]protein A and the radiolabeled bands were detected with a PhosphorImager (Molecular Dynamics). (**B**) Same as (A) except that the mitochondria were prepared from a *cox14* null mutant with an integrated plasmid expressing a Cox14p-GST fusion protein (ΔCOX14/ST32). (**C**) The bands shown in (A, B) were quantified with the PhosphorImager. The open bars represent the percentage of the corresponding protein bound to the beads, and the filled bars represent the percentage of unbound protein recovered in the supernatant fraction.

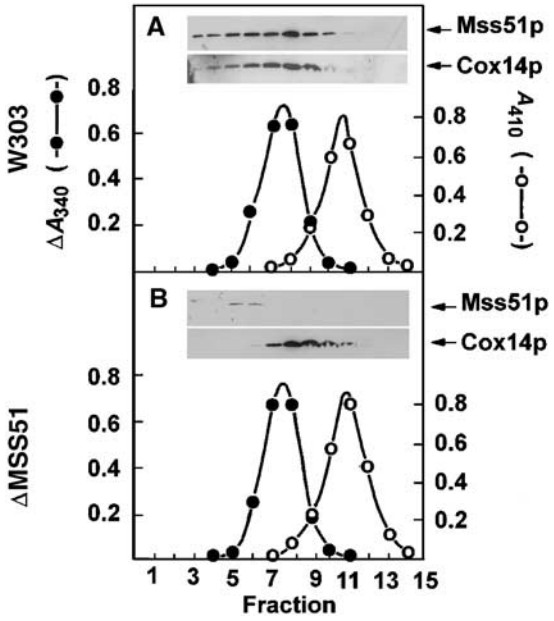

Figure 8 Sedimentation of Mss51p and Cox14p in sucrose gradients. (**A**) Mitochondria of the wild-type strain W303-1A were extracted at a protein concentration of 10 mg/ml with 1% lauryl maltoside, 20 mM Tris–HCl (pH 7.5), and 0.5 M KCl. The extract (0.4 ml) was mixed with 2.5 mg of hemoglobin and 60 μg lactate dehydrogenase and applied to 4.6 ml of a linear 7–25% sucrose gradient containing 10 mM Tris–HCl and 0.1% Triton X-100. Following centrifugation at 65 000 rpm in a Beckman SW65Ti rotor for 6 h, 14.5 fractions were collected, separated on a 12% polyacrylamide gel, transferred to nitrocellulose and probed with rabbit antiserum against Mss51p or Cox14p followed by a secondary goat peroxidase-conjugated antibody against rabbit IgG. Antibody–antigen complexes were visualized with the Super Signal reagent (Pierce Chemical Co., Rockford, IL). Hemoglobin (○---○) was estimated from absorbance at 410 nm and lactate dehydrogenase (◆--◆) was assayed by measuring oxidation of NADH at 340 nm with pyruvate as the substrate. (**B**) Same as (A) except that the mitochondria were isolated from the *mss51* null mutant aW303ΔMSS51 and the gradient was collected in 15 fractions.

behind lactate dehydrogenase with estimated masses of 130 kDa (Figure 8A). The distribution of Mss51p and Cox14p in this gradient, however, was not symmetrical, indicating the presence of higher molecular weight specie(s) (Figure 8A). The gradient of the lauryl maltoside extract of mitochondria from the *mss51* deletion mutant showed a symmetrical distribution of Cox14p with a peak at approximately the same position as Cox14p in the wild-type extract (Figure 8B). The symmetrical Cox14p peak in the gradient of the mutant extract suggests that the faster sedimenting component(s) in the wild-type extract may represent that fraction of Cox14p complexed to Mss51p. All of the Cox14p in the mutant and most of Cox14p in wild type were estimated to have a mass at least 10 times larger than the monomer. This indicates that in addition to interacting with Mss51p, Cox14p also exists as part of a larger homo or hetero-oligomeric complex.

Unlike Cox14p and Mss51p, Shy1p was not adsorbed onto the glutathione beads from mitochondrial extracts containing either Mss51p or Cox14p fused to GST (data not shown).

COX14p is a mitochondrial inner membrane protein facing the matrix

Cox14p and Mss51p were previously shown to be associated with the inner membrane of mitochondria (Glerum *et al*, 1995; Siep *et al*, 2000). To see if the localization of the two proteins is consistent with their proposed functions, we have determined their topology and solubility properties. Sonic irradiation of wild-type mitochondria solubilized cytochrome b_2, a soluble protein of the intermembrane space, but not Cox14p or Mss51p (Figure 9A). Cox14p and Mss51p, however, were solubilized with alkaline carbonate, suggesting that they are peripheral proteins (Figure 9A). In this experiment, Shy1p was recovered in the membrane fraction, confirming earlier evidence that it is an intrinsic protein of the inner membrane (Barrientos *et al*, 2002b).

Both Cox14p and Mss51p are located on the matrix side of the inner membrane as evidenced by their resistance to proteinase K in mitochondria and in mitoplasts (Figure 9B). Sco1p, an inner membrane protein previously shown to face

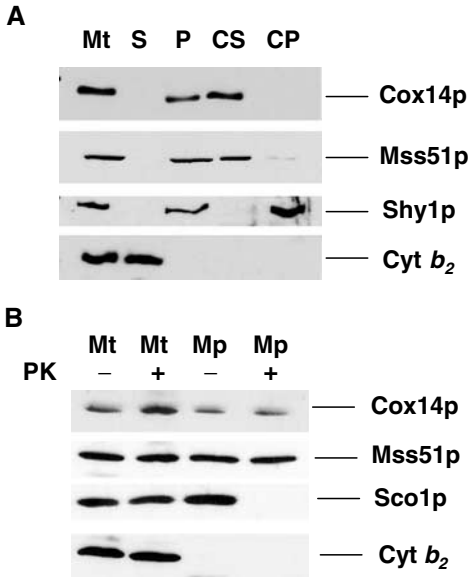

Figure 9 Localization and topology of Cox14p and Mss51p. (**A**) Mitochondria and the post-mitochondrial supernatant fractions were prepared from the wild-type strain W303-1A. A sample of mitochondria at 4 mg/ml was sonically irradiated and centrifuged at 50 000 g_{av} for 30 min. The membrane pellet was suspended in the starting volume of buffer. To 500 µl of the membranes at a protein concentration of 1 mg/ml was added 50 µl of 1 M Na_2CO_3 (pH 11.3) and 50 mM EDTA. After 30 min on ice, the sample was centrifuged at 100 000 g_{av} for 15 min at 4°C to separate the soluble from the insoluble intrinsic membrane proteins. Equivalent volumes of mitochondria (Mt), membranes (P), the supernatant obtained after centrifugation of the sonicated mitochondria (S), the carbonate supernatant (CS), and pellet (CP) were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and treated with antiserum against Cox14p as in Figure 8. Antibodies against Mss51p, Shy1p, and cytochrome b_2 (Cyt b_2) were used to monitor the conversion of mitochondria to mitoplasts and the intactness of the latter. (**B**) Mitochondria from W303-1A (Glick and Pon, 1995) at a protein concentration of 8 mg/ml in 0.6 M sorbitol and 20 mM Hepes (pH 7.5) (SH) were converted to mitoplasts (Mp) by dilution with eight volumes of 20 mM Hepes (pH 7.5). For controls, mitochondria (Mt) were diluted with eight volumes of SH. Proteinase K (prot K) was added to one-half of each sample at a final concentration of 100 µg/ml. After 60 min on ice, the reaction was stopped by addition of PMSF to a final concentration of 2 mM and the mitochondria and mitoplasts were recovered by centrifugation at 100 000 g_{av}. The pellets were suspended in SH, and proteins were precipitated by addition of 0.1 volume of 50% trichloroacetic acid and heated for 10 min at 65°C. Mitochondrial and mitoplast proteins were separated by SDS–PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with antibody against Cox14p, Sco1p, Mss51p, and cytochrome b_2. Antibody–antigen complexes were visualized as in (A).

the intermembrane space (Glerum *et al*, 1996b), was digested in the mitoplasts but not in mitochondria (Figure 9B). Disruption of the outer membrane under the hypotonic conditions used to obtain mitoplasts was confirmed by the loss of cytochrome b_2, a soluble protein marker of the intermembrane space.

Discussion

Mitochondrial translation of Cox1p, the heme-bearing subunit of COX, was previously proposed to be negatively regulated by means of its interaction with the Cox1p-specific translation factor Mss51p (Barrientos *et al*, 2002b). This is supported by the recent demonstration of an interaction between Mss51p and newly synthesized Cox1p (Perez-Martinez *et al*, 2003). In this study, we attempted to learn more about the relationship of COX assembly to Mss51-dependent synthesis of Cox1p by analyzing a wide range of different COX-deficient mutants.

In vivo assays of mitochondrial translation revealed that, with a few exceptions, labeling of Cox1p but not of the other mitochondrial gene products is 10 times less efficient in COX mutants than in wild type. Decreased labeling of Cox1p relative to other mitochondrial translation products has also been observed in *pet111* mutants that have a lesion in a translational activator of *COX2* mRNA (Poutre and Fox, 1987) and in *cox7* mutants, lacking the nuclear-encoded subunit 7 (Calder and McEwen, 1991). This phenotype cannot be accounted for by a defect in the translation system since the mutations are not in proteins (except those in *mss51*, *pet309*) related to this mitochondrial activity. Several lines of evidence also argue against a rapid turnover of Cox1p (except in the *oxa1* mutant) as an explanation for the phenotype. The decreased labeling of Cox1p is manifest even with very short pulse times. Additionally, turnover seems unlikely since *cox14* mutants, which are also compromised in COX assembly, do not display the Cox1p labeling defect. A more reasonable explanation is that expression of Cox1p is downregulated in most mutants at the translational level.

The ability of the *cox14* null mutation to suppress the Cox1p translational defect in all the mutants except the *mss51*, *pet309*, and *oxa1* mutants suggested that Cox14p either represses translation of the *COX1* mRNA or decreases the effective pool of translational activators of Cox1p such as Mss51p or Pet309p. The failure of a bypass ρ^- genome in which *COX1* is fused to the 5′-UTR of the cytochrome *b* gene (Manthey and McEwen, 1995) to restore respiration in the *cox14* mutant makes it unlikely that Cox14p acts as a translational repressor. Furthermore, overexpression of Cox14p does not diminish Cox1p synthesis.

A more plausible explanation for the effect of the *cox14* mutation on Cox1p expression is that Cox14p prevents Mss51p and/or Pet309p from promoting translation of the *COX1* mRNA in mutants unable to complete assembly of functional COX. An essential feature of the mechanism proposed here (Figure 10) is that the entry of newly translated Cox1p into the COX assembly pathway depends on the interaction of Cox1p with Cox14p and Mss51p and that when bound to Cox1p and Cox14p, Mss51p is forestalled from acting as a translational activator. Recently, Perez-Martinez *et al* (2003) provided evidence that in addition to enhancing initiation of translation of *COXI* mRNA by interacting with the 5′-UTR, Mss51p also acts on target(s) within the *COX1* coding sequence to promote translation elongation. The effect of Mss51p on elongation could be mediated either by an interaction with the mRNA or the nascent protein (Perez-Martinez *et al*, 2003). Since translational regulation by nascent chains has been reported previously (reviewed in Tenson and Ehrenberg, 2002), the latter seems to be the more plausible hypothesis. For reasons of simplicity, the scheme in Figure 10 does not distinguish between these two possibilities. Our results indicate that the interaction of Mss51p with Cox1p does not depend on Cox14p. However, the absence of Cox14p may decrease either the stability of Cox1p itself or the

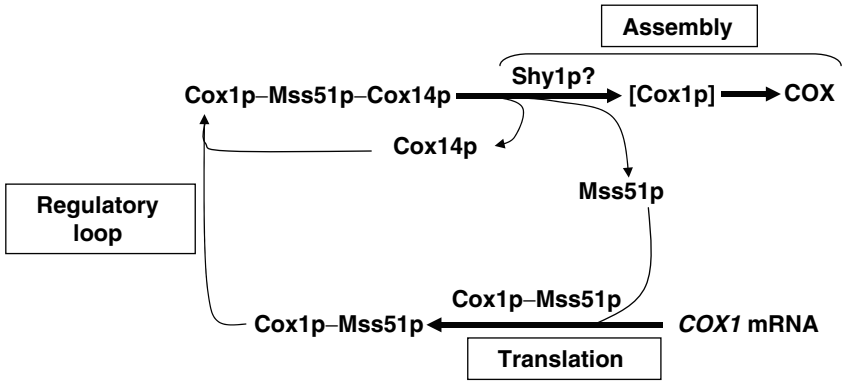

Figure 10 Model depicting Cox1p expression coupled to Shy1p-dependent cytochrome oxidase assembly. Mss51p promotes initiation and elongation of Cox1p translation (see Discussion). Cox14p and Mss51p form a ternary complex with newly synthesized Cox1p. The release of Mss51p from the ternary complex depends on a downstream Cox1p assembly step(s) perhaps involving Shy1p. According to this scheme, mutations blocking assembly trap Mss51p in the ternary complex, thereby limiting its availability for translation. In the *cox14* mutant, Mss51p is still able to complex with Cox1p. The resultant binary complex is not assembly-competent causing Cox1p to be diverted to degradation with a concomitant release of Mss51p for additional rounds of Cox1p synthesis.

Cox1p/Mss51p complex, thereby raising the effective concentration of Mss51p available for translation. This is consistent with the phenotype of *cox14* mutants and with their increased turnover of Cox1p.

According to the model proposed here, the dissociation of Mss51p from Cox1p occurs during assembly and could be catalyzed by Shy1p or other COX assembly factors. The enhancement of Cox1p translation in the presence of the *mss51* suppressor or an extra copy of wild-type *MSS51* may circumvent the Shy1p requirement and account for the partial rescue of *shy1* mutants (Barrientos *et al*, 2002b). The failure of the *cox14* null mutant to form COX and to suppress *shy1* mutants, despite normal synthesis of Cox1p, indicates that the interaction of Cox14p with Cox1p is essential for a later step in the assembly pathway.

The coupling of Cox1p synthesis to COX assembly, implicit in this regulatory scheme, is similar to the 'control by epistasy of synthesis' (CES) mechanism that has been proposed to regulate biogenesis of the photosystem complex in chloroplasts of *Chlamydomonas reinhardtii* (Wollman *et al*, 1999), and to act in translational regulation of flagellar assembly in bacteria (Aldridge and Hughes, 2002). In these systems, translation of certain subunits is contingent on the availability of their assembly partners, thereby acting as a negative feedback loop that paces translation of a subunit to its utilization during assembly of the complex (Choquet and Vallon, 2000). In the case of membrane complexes, this form of negative regulation may prevent nonproductive aggregation of unassembled hydrophobic membrane proteins by restricting their steady-state concentration.

The involvement of Mss51p and Cox14p in the regulation of Cox1p expression is supported not only by the results of the *in vivo* mitochondrial translation assays discussed above but also by the interactions of Mss51p and Cox14p with each other and with Cox1p. Reciprocal GST pull-down experiments indicate a transient (or weak) interaction (direct or indirect) of Cox14p with Mss51p. In these experiments, approximately 50% of the untagged Cox14p or Mss51p was co-adsorbed onto the glutathione–sepharose beads. Similar experiments demonstrated that nearly all of newly synthesized Cox1p is complexed to Cox14p but not to Shy1p. An interaction of Cox1p with wild-type Cox14p was also detected by co-immunoprecipitation with a Cox14p-specific antibody. The pull-down assays have also confirmed a complex of newly synthesized Cox1p with Mss51p as reported by Perez-Martinez *et al* (2003). Finally, Cox14p and Mss51p are extrinsic proteins facing the matrix side of the inner mitochondrial membrane where synthesis of Cox1p is presumed to occur. This localization is consistent with the role of Mss51p as a translational activator of *COX1* mRNA (Decoster *et al*, 1990; Siep *et al*, 2000) and with the secondary role of Mss51p and Cox14p as sensors of unassembled Cox1p.

Since COX subunits translated on mitochondrial ribosomes in cycloheximide-poisoned cells do not assemble into a native size complex (unpublished), the stability of Cox1p, Cox2p, and Cox3p in wild type under these conditions was unexpected. In contrast to wild type, newly synthesized Cox2p and Cox3p in the mutants were unstable and degraded during the chase. This was not true of Cox1p, which, despite its low initial concentration, was not reduced further, even after a long period of chase. It may be significant that the three COX subunits reached an approximately stoichiometric ratio following 2 h of chase. These observations suggest that in the wild type, the mitochondrial translation products form a protease-resistant intermediate or are sequestered in a protease-protected compartment of the membrane. The stability of Cox1p in the mutants indicates that the small amount of the subunit synthesized in these strains, as well as a similar fraction of Cox2p and Cox3p, is able to attain a protected state similar to that seen in wild type. Most of Cox2p and Cox3p in the mutants, however, are susceptible to proteolytic degradation. This also applies to Cox1p in the single and double *cox14* mutants. The extra Cox1p made in these strains, as a result of impaired translational regulation, is prevented from being converted to a protease-resistant form because of its failure to be integrated into an assembly intermediate or incorporated into a protected compartment.

At present, it is not clear if translation of Cox1p in other organisms is also subject to regulation by downstream events. Of the more than dozen yeast nuclear genes governing different post-translational events in COX assembly, half are currently known to have human homologues (Barrientos

et al, 2002a). Shy1p is a conserved protein found in all eukaryotes and some prokaryotes (Poyau *et al*, 1999). Homologues of Cox14p, however, have only been found in *Kluyveromyces lactis* (Fiori *et al*, 2000), and of Mss51p in *Schizosaccharomyces pombe* and in *Neurospora crassa*. This does not necessarily exclude the possibility that they may exist in mammalian and other genomes but have not yet been recognized because of their smaller size and/or divergent sequences. For example, the products of yeast *PET309* and human *LRPPRC* (*LRP130*), responsible for the Canadian form of Leigh's syndrome (Mootha *et al*, 2003), display very weak sequence similarity, even though they both bind to mitochondrial RNAs and are essential for COX expression. It is also possible that translational activators such as Pet309p and Mss51p have more than one function (i.e. mRNA metabolism, membrane insertion of the newly synthesized protein), only some of which are conserved among different organisms. Understanding the nature and players involved in the mechanism regulating COX assembly in yeast will help to identify their functional homologues in humans.

Materials and methods

Strains and media

The genotypes and sources of the *Saccharomyces cerevisiae* strains carrying null alleles of COX-related genes are listed in Table I.

In vivo and in organello mitochondrial protein synthesis

Mitochondrial gene products were labeled with [^{35}S]methionine in whole cells at 30°C in the presence of cycloheximide (Barrientos *et al*, 2002b). For *in organelle* translation, mitochondria were prepared by the method of Herrmann *et al* (1994) and labeled with [^{35}S]methionine as described (Hell *et al*, 2000). Equivalent amounts of total cellular or mitochondrial proteins were separated by SDS–PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to an X-ray film.

Preparation of antibodies to Cox14p

Antibodies were obtained against Cox14p expressed from a *trpE* fusion gene. The full coding region of the *COX14* gene was PCR amplified with the primers 5′-GGCGGATCCAT-GTCCAAATAC GCTTGG and 5′-GGCAAGCTTGGAACCAGC-ACACTACGT. The amplified *Bam*HI–*Hin*dIII fragment was fused in-frame to the amino-terminal half of *trp*E in pATH21. *Escherichia coli* transformed with this plasmid expresses a fusion protein of about 45 kDa, constituting

Table I Genotypes and sources of yeast strains carrying null alleles of COX-related genes

Strain	Genotype	Source
Structural subunits		
W303ΔCOX4	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox4::URA3*	Glerum and Tzagoloff (1997)
W303ΔCOX5a	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox5a::HIS3*	Glerum and Tzagoloff (1997)
W303ΔCOX6	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox6::URA3*	Glerum and Tzagoloff (1997)
W303ΔCOX7	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox7::URA3*	This study
W303ΔCOX9	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox9::URA3*	This study
M10-150	*cox1*	Tzagoloff *et al* (1975)
M9-94	*cox2*	Tzagoloff *et al* (1975)
M9-3	*cox3*	Tzagoloff *et al* (1975)
Cytochrome c maturation		
W303ΔCYC3	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcyc3::URA3*	This study
COX1 expression		
W303ΔPET309	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet309::HIS3*	Glerum and Tzagoloff (1997)
W303ΔMSS51	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δmss51::HIS3*	Barrientos *et al* (2002b)
Maturation of CuA or CuB centers		
W303ΔCOX17	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox17::TRP1*	Glerum *et al* (1996a)
W303ΔSCO1	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δsco1::URA3*	Glerum *et al* (1996b)
W303ΔCOX11	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox11::HIS3*	Tzagoloff *et al* (1990)
COX2 expression		
W303ΔOXA1	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δoxa1::HIS3*	Hell *et al* (2000)
W303ΔCOX18	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox18::URA3*	Souza *et al* (2000)
W303ΔPET111	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet111::HIS3*	Barros and Tzagoloff (2002)
W303ΔIMP1	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δimp1::HIS3*	This study
W303ΔIMP2	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δimp2::URA3*	Barros *et al* (2001)
Heme biosynthesis		
W303ΔCOX10	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox10::HIS3*	Nobrega *et al* (1990)
W303ΔCOX15	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox15::HIS3*	Glerum *et al* (1997)
Assembly/unknown		
W303ΔPET117	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet117::HIS3*	Barros and Tzagoloff (2002)
C8/L1	*leu2 pet117*	Tzagoloff and Dieckmann (1990)
W303ΔSHY1/U2	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δshy1::URA3*	Barrientos *et al* (2002b)
W303ΔPET191	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δpet191::HIS3*	This study
W303ΔCOX14	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox14::TRP1*	This study
W303ΔCOX16	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox14::URA3*	Carlson *et al* (2003)
W303ΔCOX19	*ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox19::URA3*	Nobrega *et al* (2002)

The part headings indicate the functional category of the deleted gene products.
All null mutations have been created or are available in both, a and α, mating types.

most of the insoluble proteins of the cells. This fraction was dissolved in 2% SDS, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol, and 20 μg/ml PMSF and was further purified on a Bio-Gel A 0.5 column developed with a buffer containing 0.1% SDS, 10 mM Tris–HCl (pH 7.5), and 0.1 M NaCl. Fractions enriched for the fusion protein were pooled, and precipitated with acetone precipitation. The precipitated protein was dissolved in 0.2% SDS and used to raise antibodies in rabbits.

Construction of GST chimeras

The construction of plasmids containing chimeric *SHY1-GST, MSS51-GST,* and *COX14-GST* genes was performed in two steps. First, the *GST* gene was amplified by PCR and, second, cloned in-frame to the corresponding genes into plasmids already containing them.

The *GST* gene plus a thrombin cleavage site at its N-terminus was amplified from pGEX-3X (Amersham Biosciences Corp., Piscataway, NJ) with primers 5′-GGCTGCAGCTGGTTCCGCGTG GATCCGGAGGAATGTCCCCTATACTAGGT and 5′-CCGGGAGCTCG GATCCACGCGGAACCAGATCC or with primers 5′-GGGGTACCTACT GGTTCCGCGTGGATCCGGAGGAATGTCCCCTATACTAGGT and 5′-CCGGGAG-CTCGGATCCACGCGGAACCAGATCC. The ~600-bp products were digested with *Pst*I/*Sac*I and *Kpn*I/*Sac*I respectively and kept until used for further cloning.

To prepare a *SHY1-GST* chimeric gene with a thrombin cleavage site, a portion of the *SHY1* gene, starting at the *Bam*HI site, was amplified with primers 5′-GGCGAGCTCCTGCAGATATTTCCTT GAATGCTTC and 5′-TGGGCGAAAA-AGGATCCAAATTC using the plasmid pG91/T1 (Mashkevich *et al*, 1997). The amplicon was cloned into the integrative plasmid pG91/ST17 (Mashkevich *et al*, 1997) from which the *Bam*HI/*Sac*I had been removed to yield the plasmid pG91/ST60. The previously amplified *GST* gene was cloned into this plasmid as *Pst*I–*Sac*I, in-frame with the 3′ end of *SHY1* to obtain pG91/ST62.

To construct the *COX14-GST* chimeric gene with a thrombin cleavage site, the *COX14* gene was amplified with the primers 5′-GGCGGAATTCAACTAATGATTGG and 5′-GGCAAGCTTGAGCTCC TGCAGCTCGGTAGGAGGAGGTGCAG using the plasmid pG93/T1 (Glerum *et al*, 1995) as the template, and cloned as an *Eco*RI/*Hin*dIII fragment into YIp352. The previously amplified *GST* gene was cloned into this plasmid as *Pst*I–*Sac*I, in-frame with the 3′ end of *COX14* to obtain pG93/ST32.

The *MSS51-GST* chimeric gene was constructed by first cloning *MSS51* as a *Kpn*I/*Sph*I fragment in YIp352. The sequence coding for *GST* was then cloned into this plasmid as a *Pst*I–*Sac*I fragment, in-frame with the 3′ end of *MSS51* to obtain pG96/ST13.

Miscellaneous procedures

Standard procedures were used for the preparation and ligation of DNA fragments, and for transformation and recovery of plasmid DNA from *E. coli* (Maniatis *et al*, 1982). 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 chromosomal DNA.

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