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Interactions among *COX1*, *COX2*, and *COX3* mRNA-specific Translational Activator Proteins on the Inner Surface of the Mitochondrial Inner Membrane of *Saccharomyces cerevisiae*

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The core of the cytochrome *c* oxidase complex is composed of its three largest subunits, Cox1p, Cox2p, and Cox3p, which are encoded in mitochondrial DNA of *Saccharomyces cerevisiae* and inserted into the inner membrane from the inside. Mitochondrial translation of the *COX1*, *COX2*, and *COX3* mRNAs is activated mRNA specifically by the nuclearly coded proteins Pet309p, Pet111p, and the concerted action of Pet54p, Pet122p, and Pet494p, respectively. Because the translational activators recognize sites in the 5′-untranslated leaders of these mRNAs and because untranslated mRNA sequences contain information for targeting their protein products, the activators are likely to play a role in localizing translation. Herein, we report physical associations among the mRNA-specific translational activator proteins, located on the matrix side of the inner membrane. These interactions, detected by coimmune precipitation and by two-hybrid experiments, suggest that the translational activator proteins could be organized on the surface of the inner membrane such that synthesis of Cox1p, Cox2p, and Cox3p would be colocalized in a way that facilitates assembly of the core of the cytochrome *c* oxidase complex. In addition, we found interactions between Nam1p/Mtf2p and the translational activators, suggesting an organized delivery of mitochondrial mRNAs to the translation system.

INTRODUCTION

Translational control and mRNA localization, achieved via a variety of mechanisms, are important for the delivery of certain cytoplasmically synthesized proteins to their functional destinations within cells of animals (Johnstone and Lasko, 2001; Palacios and Johnston, 2001), plants (Choi *et al.*, 2000), and yeast (Zoladek *et al.*, 1995; Lithgow *et al.*, 1997; Corral-Debrinski *et al.*, 2000). In general, these strategies seem to reinforce targeting information present within the protein products themselves. In *S. cerevisiae*, for example, nuclearly coded mRNAs for several mitochondrial proteins bearing mitochondrial import signals seem to be translated preferentially by cytoplasmic ribosomes tightly associated with the organelles, facilitating their localization (Marc *et al.*, 2002). This targeting is dependent upon signals in the mRNA 3′-untranslated regions.

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Translation within the mitochondrial matrix of most, if not all, mRNAs encoded in mitochondrial DNA depends upon mRNA-specific translational activators that recognize targets in the mRNA 5′-untranslated leaders (UTLs) and seem to mediate mRNA interactions with mitochondrial ribosomes (Fox, 1996a). All but one of the major proteins encoded by yeast mitochondrial genes are integral membrane proteins that are assembled with nuclear gene products to form respiratory chain complexes in the inner membrane (Pon and Schatz, 1991). The core of the cytochrome *c* oxidase complex of mammals and yeast comprises three mitochondrially coded subunits, Cox1p, Cox2p, and Cox3p, and is surrounded by imported subunits coded by nuclear genes (Tzagoloff and Dieckmann, 1990; Tsukihara *et al.*, 1996). Translation of each mitochondrially coded mRNA is specifically activated by distinct nuclear gene products: Pet309p for *COX1* (Manthey and McEwen, 1995), Pet111p for *COX2* (Mulero and Fox, 1993a,b), and Pet54p, Pet122p, and Pet494p for *COX3* (Costanzo and Fox, 1988; Brown *et al.*, 1994). Pet309p, detected as an overproduced epitope-tagged protein, is an integral inner membrane protein partially exposed on the intermembrane space side (outside) (Man-

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
MCC100	*MATa ura3-52 pet494-41* [ρ^+]	M. C. Costanzo
CAB21-2	*MATa ura3-52 PET494::3xHA* [ρ^+]	This study
CAB13	*MATa ade2 ura3-52 trp1-Δ1 pet122-6* [ρ^+]	This study
CAB14	*MATa ade2 ura3-52 PET122::3xHA::TRP1* [ρ^+]	This study
CAB30	*MATa ura3-52 PET494::3xHA PET122::3xHA* [ρ^+]	This study
SN25	*MATa PET494::3xHA PET122::3xHA PET54::3xMYC* [ρ^+]	This study
SN28	*MATα ura3-52 trp1-Δ1 [rho$^+$] PET54::3xMYC* [ρ^+]	This study
SN32	*MATa ura3-52 PET494::3xHA PET122::3xHA PET309::3xMYC* [ρ^+]	This study
SN33	*MATa ura3-52 PET494::3xHA PET122::3xHA PET111::3xMYC* [ρ^+]	This study
PJ69-4A	*MATa ura3-52 leu2-3, 112 trp1-901 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ* [ρ^+]	James *et al.*, (1996)
PTY11	*MATα ura3-52 trp1-Δ1* [ρ^+]	Green-Willms *et al.*, (1998)
SB09	*MATα ade2 PET309::3xHA ura3-52* [ρ^+]	S. A. Broadley
SN24	*MATα ade2 PET309::3xHA nam1Δ::URA3* [ρ^+]	This study

they *et al.*, 1998). Pet111p, detected as an epitope-tagged protein at wild-type levels, is an integral inner membrane protein facing the matrix (Green-Willms *et al.*, 2001). Pet54p is a peripheral inner membrane protein, whereas Pet122p and Pet494p, detected as overproduced proteins, behaved like integral inner membrane proteins (McMullin and Fox, 1993). The topology of the *COX3* mRNA-specific proteins has not been previously investigated.

The fact that several mRNA-specific translational activator proteins were found to be bound to the inner membrane suggested that they could localize translation to sites where the mitochondrial gene products could be efficiently assembled into respiratory complexes (Costanzo and Fox, 1990; Michaelis *et al.*, 1991; McMullin and Fox, 1993; Fox, 1996a). Consistent with this hypothesis, topological information required for efficient assembly of two mitochondrially coded subunits of the cytochrome *c* oxidase complex, Cox2p and Cox3p, has been shown to reside in untranslated portions of their mRNAs (Sanchirico *et al.*, 1998). In addition to their apparent role in localizing translation on the matrix side of the inner membrane, the *COX2* and *COX3* mRNA-specific translational activators are also present at levels that limit expression of their target mitochondrial genes (Steele *et al.*, 1996; Green-Willms *et al.*, 2001).

Although it is easy to imagine a biological rationale for using a single mechanism to both regulate the expression level of an organellar gene and to target its product to the inner membrane, possible rationales for mRNA-specific functions are less obvious. Why should the mRNAs specifying the three core subunits of the cytochrome *c* oxidase complex, Cox1p, Cox2p, and Cox3p, require three distinct nuclearly encoded translational activators? One attractive hypothesis is that these mRNA-specific translational activators could be organized on the surface of the inner membrane such that they would promote adjacent translation of the *COX1*, *COX2*, and *COX3* mRNAs, and thereby facilitate assembly of the cytochrome oxidase core.

A clear prediction of this hypothesis is that the translational activators for the three cytochrome oxidase subunits should physically interact with each other. In this study, we have tested this prediction by two-hybrid analysis and coimmune precipitation experiments. We found evidence for a network of interactions among these translational activator proteins, suggesting that they could be organized in the mitochondrial inner membrane to colocalize synthesis of the three core cytochrome *c* oxidase subunits. Furthermore, we found evidence that Nam1p/Mtf2p, a protein involved in mRNA transactions that interacts with the mitochondrial RNA polymerase (Lisowsky and Michaelis, 1989; Wallis *et al.*, 1994; Rodeheffer *et al.*, 2001; Bryan *et al.*, 2002), also interacts with the translational activators. This suggests an organized flow of information from mitochondrial DNA to the inner mitochondrial membrane.

MATERIALS AND METHODS

Yeast Strains, Media, and Standard Methods

Saccharomyces cerevisiae strains used in this study are listed in Table 1. All strains are isogenic to the wild-type strain D273-10B (ATCC 25657) except PJ69-4a (James *et al.*, 1996). Cells were grown in rich medium YPD or YPR (1% yeast extract, 2% bacto-peptone, 2% glucose or 2% raffinose) and in nonfermentable medium YPEG (1% yeast extract, 2% bacto-peptone, 20 mg adenine/l, 3% ethanol, 3% glycerol). Synthetic complete (SC) media have been described previously (Sherman, 1991) except that nutritional supplements lacking specific factor(s) were purchased from Bio 101 (Vista, CA). Transformation of plasmids and polymerase chain reaction (PCR) products into yeast cells were accomplished by using EZ-Transformation kit (Zymo Research, Orange, CA). To construct the *nam1* deletion strain SN24, a disruption cassette containing the *URA3* gene flanked by 45 base pairs of sequence homologous to the *NAM1* coding region was amplified by PCR, purified, and transformed into SB09. The transformants were selected on SC-uracil media and then printed on YPEG plates to identify cells incapable of respiratory growth, because *NAM1* is essential for respiration. Deletion of *NAM1* was confirmed by PCR.

Epitope Tagging

The coding region of *PET122* from +170 to +961 base pairs (relative to the ATG start codon) was amplified by PCR by using upstream primer 5′-GGG AAT TCC CAT GCC GAC ACT ATA GC-3′ and downstream primer 5′-CCC CAT GGT GTT GAT TTC AAA TCC TCT-3′. The amplified DNA fragment was subcloned at *Nco*I-*Eco*RI sites of the p3XHA plasmid (Tyers *et al.*, 1992). The resulting plasmid, pPET122HA, contains an insertion of the hemagglutinin (HA)-cassette (encoding three tandem copies of HA-epitope) at the C terminus of the *PET122.* pPET122HA was linearized with *Nru*I and

then transformed into strain CAB13. Trp^+ transformants were printed on YPEG plates to check restoration of respiratory growth. The integration of *PET122-HA* was confirmed by PCR for strain CAB14.

Pet494p is inactivated by some modifications to its C terminus (our unpublished data). We therefore inserted an HA-cassette at +358 base pairs relative to *PET494* ATG start codon, a region of the coding sequence known to tolerate sequence changes (Costanzo *et al.*, 1986). A 4.7-kb *Hin*dIII-*Eco*RI fragment containing full-length *PET494* was cloned in pBluescript KS (Stratagene, La Jolla, CA) that had the polylinker *Xho*I site was removed by digestion with *Apa*I and *Sal*I, followed by Klenow treatment and religation. The HA-cassette from p3XHA was amplified by PCR by using upstream primer 5′-GGA ATT CCG GCT CGA GGC ACT GAG CAG CGT AAT CTG G-3′ and downstream primer 5′-GGA ATT CCG GCT CGA GTA CCC ATA CGA TGT TCC TG-3′ and then cloned into pCB4 at the *Xho*I site. The resulting plasmid pPET494HA contains an in-frame fusion of the HA-cassette with the coding region of *PET494* at +358 base pairs (relative to ATG initiation codon). Recombinant plasmid pPET494HA was digested with *Hin*dIII and *Eco*RI and a 2.9-kb insert containing HA-tagged *PET494* was purified and cotransformed with YEp24 (Botstein *et al.*, 1979) into MCC100. Transformants were selected on SC-uracil plates and subsequently printed on YPEG plates to score restoration of functional *PET494*. Integration of the *PET494-HA* fragment was verified by PCR in CAB21-2.

To incorporate three copies of HA or MYC tags at the C terminus of other proteins, an *HA-URA3-HA* or *MYC-URA3-MYC* cassette flanked by 45 base pairs of sequence homologous to the gene of interest was amplified by PCR by using the appropriate primer pair (Schneider *et al.*, 1995). The resulting PCR products were transformed into desired strains and integration of the MYC or HA-cassette at the desired genomic locus was identified by PCR. Transformants were streaked on medium containing 5′-fluoroorotic acid to select for the loss of selected marker *URA3.* In-frame fusion of the *HA* or *MYC*-cassette was confirmed by sequencing in each case.

Two-Hybrid Analysis

The multicopy expression vectors pGBDU-C1, pGAD-C1 (James *et al.*, 1996), pGAD424, pGBT9 (Bartel *et al.*, 1993), and pGAD2F (Chien *et al.*, 1991) used in this study have been described elsewhere. The *PET309* coding region was cloned in the pGADC1 and pGBDUC1 two-hybrid vectors at *Sal*I site to create pSN20 and pSN21 containing in-frame fusion of *PET309* coding region with the activation domain (AD) and DNA-binding domain (BD) of *GAL4*, respectively. The partial *PET111* coding region (from +112 to +2434 relative to the *PET111* initiation codon) was cloned into pGBT9 at the *Bam*HI site to create pNSG5, containing an in-frame fusion of the *PET111* coding region with the BD of *GAL4* (Green-Willms, unpublished data). The *Xma*I-*Pst*I fragment from pNSG5 was cloned into pGAD424. The resulting plasmid, pSCS2, contains an in-frame fusion of the *PET111* coding region to the AD of *GAL4*.

Plasmids carrying coding regions of the *PET54* (pNGB8), *PET122* (pNGB11), and *PET494* (pNGB39) were described previously (Brown *et al.*, 1994). DNA fragments similar to those present in the pNGB8, PNGB11, and pNGB39 were cloned into the binding domain plasmid pGBT9 at the *Bam*HI site. The resulting plasmids pNGB67 and pNGB68 contain fusion of the BD of *GAL4* with the full-length coding region of *PET54* or *PET122*, respectively. pNGB70 expresses a hybrid protein consisting of the Gal4p BD fused to an amino terminal truncated form of the Pet494p (lacking 146 residues). The correct orientation and in-frame translational fusion in each case were confirmed by sequencing. Plasmids pACT and pACT-NAM1 were gifts from Dr. G.S. Shadel (Emory University, Atlanta, GA).

All AD plasmids used in this study carried the *LEU2* marker gene. The binding domain plasmids based on pGBT9 carried tryptophan (*TRP1*) selection marker, and pGBDU-C1 based plasmids carried *URA3* marker gene. To test interactions a plasmid pair was transformed into the yeast two-hybrid strain PJ69-4a and double transformants were selected on SC media lacking appropriate nutrients depending upon the selection markers on the two plasmids. The growth of the double transformants was tested on SC-histidine containing 2.5 mM 3-aminotriazole and SC-adenine.

Mitochondrial Isolation, Purification, Subfractionation, and Western Analysis

Mitochondria were prepared from late exponential phase cells grown at 30°C in YPR. Mitochondrial isolation, purification, mitoplasting, proteinase K treatment, SDS-PAGE, and Western analyses were carried out as described previously (Glick, 1995; Glick and Pon, 1995; He and Fox, 1997, 1999). Anti-HA-horseradish peroxidase (HRP) (3F10) and anti-MYC-HRP (9E10) were purchased from Roche Diagnostics (Indianapolis, IN). Polyclonal anti-Nam1p was a gift from N. Bonnefoy (CNRS, Gif-sur-Yvette, France), and anti α-ketoglutarate dehydrogenase was a gift from B. Glick (University of Chicago, Chicago, IL). The enhanced chemiluminescence plus detection system (Amersham Biosciences, Piscataway, NJ) was used for detection of proteins on Western blots.

Cross-Linking and Coimmunoprecipitations

Mitochondria (500 μg-1 mg of protein) were resuspended at 1 mg/ml in cross-linking buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.6 M sorbitol, 1 mM EDTA, and protease inhibitors cocktail (Roche Diagnostics). Samples were incubated with 1 mM dithiobis(succinimidylpropionate) (DSP) or 2 mM dithio-bis-maleimidoethane (DTME)-membrane permeable, thiol-reversible chemical cross-linkers, at 25°C for 30 min or 1 h, respectively, followed by addition of 100 mM Tris-HCl, pH 8.0 to quench the excess cross-linker. The mitochondria were washed once in 0.6 M sorbitol 50 mM HEPES pH 7.4 and then gently resuspended in solubilization buffer. In experiments where digitonin was used mitochondria were solubilized (1 mg/ml) in immunoprecipitation (IP) buffer 1 (150 mM KOAc, 50 mM HEPES, pH 7.4, 2 mM MgOAc, 2 mM ATP, protease inhibitors cocktail, 1% digitonin) at 4°C for 30 min. Alternatively, mitochondria were solubilized (2.5 mg/ml) with Triton X-100 by using IP buffer 2 (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, protease inhibitors cocktail) at 4°C for 30 min. The soluble fraction was clarified by centrifugation at 80,000 $\times$ *g* for 20 min and supernatant was incubated with agarose beads (Sepharose CL-4B; Sigma-Aldrich, St. Louis, MO) at 4°C for 1 h under gentle shaking conditions. The supernatant was then incubated with either anti-HA (3F10) affinity matrix (Roche Diagnostics) or anti-MYC (9E10)-agarose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C under gentle shaking conditions. Precipitates from digitonin-solubilized mitochondria were washed three times with the IP buffer 1 and once with wash buffer 1 (150 mM KOAc, 50 mM HEPES, pH 7.4, 2 mM MgOAc, 2 mM ATP, 0.1% NP-40, 0.1% Triton X-100). Precipitates from Triton X-100–solubilized mitochondria were washed four times with wash buffer 2 (50 mM HEPES, pH 7.4, 50 mM NaCl, 10% glycerol, protease inhibitors, 0.05% NP-40, 0.1% Triton X-100). Proteins were eluted from beads with SDS sample buffer containing 150 mM dithiothreitol by boiling for 3 min and were analyzed by Western blotting.

RESULTS

Interacting Components of the COX3 mRNA-specific Activator Are on Inner Surface of Mitochondrial Inner Membrane

We have previously found that both Pet494p and Pet122p were associated with the mitochondrial inner membrane

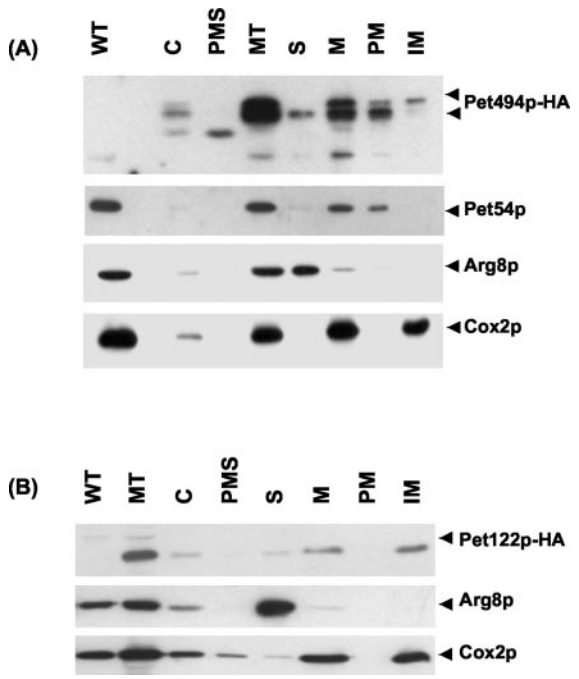

Figure 1. COX3 mRNA-specific activators Pet54p, Pet122p, and Pet494p are bound to the mitochondrial inner membrane. Mitochondria were purified from yeast strains bearing *PET494-HA* (CAB21–2) (A) and *PET122-HA* (CAB14) (B). Aliquots (50 μg) of total cell extract (C), postmitochondrial supernatant (PMS), and total mitochondrial proteins (MT), as well as total mitochondrial protein from an untagged wild-type strain PTY11 (WT) were analyzed on Western blots along with submitochondrial fractions. Mitochondrial proteins were separated into soluble (S) and membrane fractions (M). Membranes were extracted with alkaline carbonate to separate alkaline-soluble peripheral (PM) and alkaline-insoluble integral (IM) membrane proteins. The Western blots were probed with anti-HA-HRP to detect HA-tagged Pet122p or Pet494p. The filters were stripped and reprobed with antibody against Pet54p; Cox2p, an integral membrane; and Arg8p, a soluble matrix protein.

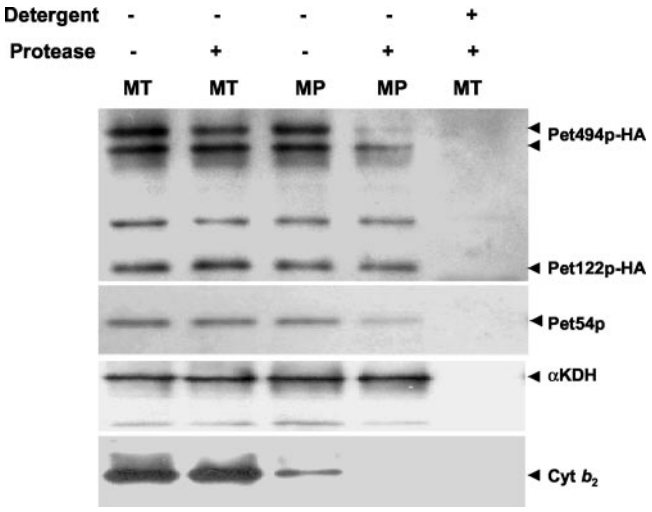

Figure 2. Submitochondrial localization of the *COX3* mRNA translational activator proteins Pet54p, Pet122p, and Pet494p. Mitochondria were isolated from yeast a strain expressing HA-tagged form of Pet122p-HA and Pet494p-HA (CAB30). Purified mitochondria (MT) and mitoplasts (MP) were subjected to digestion with proteinase K (100 μg/ml) in presence (+) and in absence (−) of 1% octyl-glucoside, a nonionic detergent. Treated samples were analyzed by Western blotting by using anti-HA-HRP to detect Pet122p-HA and Pet494p-HA. Filters were stripped and reprobed with antisera against Pet54p, the soluble intermembrane space protein cytochrome b_2 (Cyt b_2) and the soluble matrix marker α-ketoglutarate dehydrogenase (αKDH).

when overproduced (McMullin and Fox, 1993). However, firm conclusions regarding localization of a protein and its in vivo associations cannot be drawn from studying cells overproducing it. We therefore tagged Pet122p and Pet494p with the HA-epitope by modification of their respective chromosomal genes (see MATERIALS AND METHODS), and examined the submitochondrial location of these proteins along with wild-type Pet54p. Purified mitochondria were fractionated after disruption by osmotic shock and sonication to separate membrane proteins from soluble proteins. Western analysis revealed that Pet122p-HA and most of the Pet494p-HA were recovered in the pellet (Figure 1, A and B), indicating membrane association of these proteins. Pet494p-HA is present as a series of species apparently generated by proteolysis. Full-length Pet494p-HA (~57 kDa) is present only in the membrane fraction; however, a small proportion of a shorter form (~53 kDa) is also present in the soluble fraction. Cox2p and Pet54p were also found in the membrane fraction, whereas Arg8p fractionated with the soluble proteins as expected (Figure 1, A and B). In previous similar experiments, we recovered roughly half of Pet54p in the soluble fraction (McMullin and Fox, 1993). However, we are unable to reproduce this result for unknown reasons.

The membrane fraction was then extracted with alkaline sodium carbonate to separate peripheral membrane proteins from integral membrane proteins. Pet54p and most of the Pet494p-HA could be solubilized with alkaline carbonate (Figure 1A). However, a significant fraction of full-length Pet494p-HA (Figure 1A), and all of the Pet122p-HA (Figure 1B) could not be solubilized by alkaline carbonate extraction, indicating that they are integral membrane proteins. The known integral membrane protein Cox2p remained completely associated with the membranes in this experiment, whereas the soluble matrix protein Arg8p did not (Figure 1, A and B).

Protease protection experiments were used to determine the submitochondrial location of the three proteins of the *COX3* mRNA-specific activator. Mitochondria were prepared from a yeast strain (CAB30) expressing both Pet494p-HA and Pet122p-HA from their chromosomal genes. Proteinase K treatment of detergent-solubilized mitochondria eliminated detectable Pet54p, Pet122p-HA, and Pet494p-HA, showing that none of these proteins are protected by a stable protein complex (Figure 2). However, these three proteins were protected from proteinase K when the mitochondria were intact. Protease treatment of mitoplasts, whose outer membranes were ruptured by osmotic shock, had relatively little effect on either Pet122p-HA or Pet54p, indicating that they are completely within the inner membrane (Figure 2). However, protease treatment of mitoplasts destroyed almost completely the full-length Pet494p-HA, while leaving a shorter form undegraded (Figure 2).

This result suggests that the full-length integral membrane protein Pet494p is partially exposed on the outer surface of the inner membrane, probably at the N terminus. However, the HA-epitope was inserted in-frame with the *PET494* gene at codon 120 (see MATERIALS AND METHODS), because changes in the C terminus of Pet494p lead to respiratory defects (our unpublished data), making interpretation of the topology difficult. Pet122p was HA tagged at its C terminus, which remains protected in mitoplasts. The soluble intermembrane space protein cytochrome b_2 was largely lost during mitoplasting, whereas the soluble matrix protein α-ketoglutarate dehydrogenase remained completely protected (Figure 2).

Two-hybrid analysis had previously suggested that Pet54p, Pet122p, and Pet494p interact to form a *COX3* mRNA-specific activator complex (Brown *et al.*, 1994). To confirm these interactions, we constructed a strain (SN25) expressing Pet122p-HA, Pet494p-HA, and Pet54p-Myc from their chromosomal loci and carried out coimmune precipitation experiments. Mitochondria purified from this strain were treated with the cross-linker DSP before solubilization with 1% digitonin (see MATERIALS AND METHODS). These soluble extracts were incubated with anti-HA antibody coupled to beads, and the resulting immunoprecipitates were examined by Western analyses by using anti-Myc, and anti-HA, antibodies (see MATERIALS AND METHODS). As shown in Figure 3A, Pet54p-Myc was coimmunepreciptated with Pet122p-HA and Pet494p-HA, but was not precipitated from control extracts of mitochondria lacking HA tags on Pet122p and Pet494p (Figure 3A). In a converse experiment in which anti-Myc antibody coupled to agarose beads was used to precipitate Pet54p-Myc, specific coimmune precipitation of Pet122p-HA and Pet494p-HA was also observed (Figure 3B).

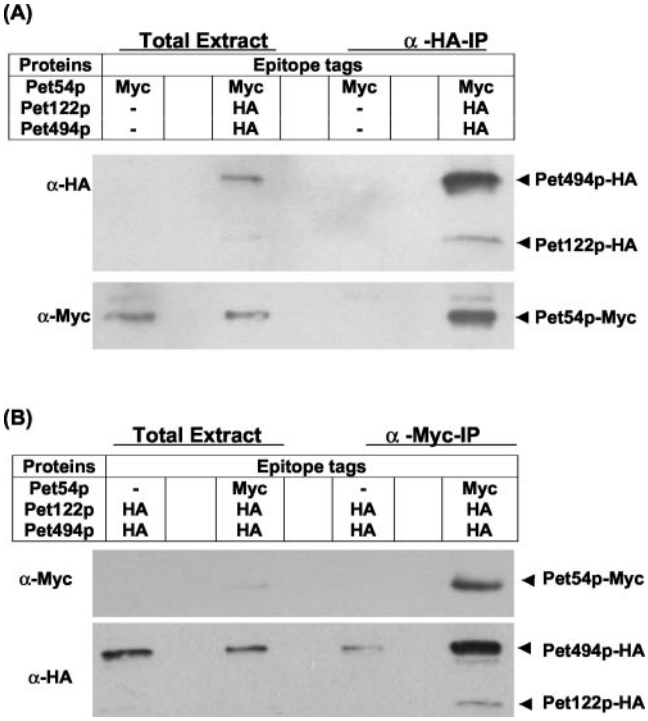

Figure 3. Coimmune precipitation of the *COX3* mRNA-specific activator proteins Pet54p, Pet122p, and Pet494p. Purified mitochondria containing the indicated epitope-tagged proteins were treated with 1 mM DSP, a membrane-permeable cross-linker at 25°C for 30 min followed by solubilization with 1% digitonin at 4°C for 30 min (see MATERIALS AND METHODS). After a clarifying spin, solubilized extracts (from 400 μg of mitochondria) were incubated with anti-HA affinity matrix (A) or with anti-Myc-agarose conjugate (B) for 4 h at 4°C under gentle shaking conditions and immunoprecipitates were analyzed by Western blotting with anti-HA-HRP and anti-Myc-HRP (see MATERIALS AND METHODS). Solubilized mitochondrial proteins (20 μg) were loaded in total extract lanes. Strains used were SN25, SN28, and CAB30 (Table 1).

Interactions between COX1 *and* COX3 *mRNA-specific Translational Activator Proteins*

We first sought evidence for interactions of the *COX1* mRNA-specific translational activator Pet309p with the *COX3* translational activators Pet54p, Pet122p, and Pet494p by using the yeast two-hybrid system. Appropriate plasmid pairs were transformed into test strain PJ69-4a (James *et al.*, 1996), and activation of the *HIS3* and *ADE2* reporter genes was monitored. Cells containing Pet309p fused to a DNA binding domain of Gal4p alone, and Pet54p or Pet122p fused to an activating domain of Gal4p alone did not express the reporters (Figure 4). However, reporter expression was observed when the Pet309p fusion protein was expressed with either the Pet54p or the Pet122p fusion protein (Figure 4). No two-hybrid interactions were detected between Pet309p and Pet494p, nor were any self-interactions detected in our experiments (our unpublished data).

We further explored these interactions by coimmune precipitation. We constructed a strain (SN32) expressing Pet122p-HA, Pet494p-HA, and Pet309p-Myc from chromosomal genes. Mitochondria from this strain were incubated with 2 mM DTME before solubilization with 1% digitonin (see MATERIALS AND METHODS). The soluble extract was precipitated with anti-Myc-agarose and the precipitate analyzed by a Western probed with anti-HA antibody. Roughly 5% of the Pet309p-Myc was precipitated, and Pet494p-HA coimmunepreciptated with approximately equal efficiency (Figure 5). The yield of coimmunepreciptated Pet494p-HA was approximately fivefold lower when the cross-linker was omitted (our unpublished data). Taken together with the two-hybrid results, the coimmune precipitations strongly suggest an association between the *COX1* and *COX3* mRNA-specific translational activators.

Interactions between COX2 *and the* COX3 *mRNA-specific Activators*

We tested for interactions of the *COX2* mRNA-specific activator Pet111p with the *COX3* activators Pet54p, Pet122p, and Pet494p by two-hybrid and coimmune precipitation experiments. An N-terminally truncated derivative of Pet111p fused to a DNA binding domain failed to activate reporter gene expression, but the combination of the Pet111p fusion and a Pet54p-activating domain fusion did, indicating physical interaction between Pet111p and Pet54p (Figure 4). Cells expressing the Pet111p fusion and an N-terminally truncated Pet494p-activating domain fusion (expressed from pNGB39 (Brown *et al.*, 1994)) were able to activate only

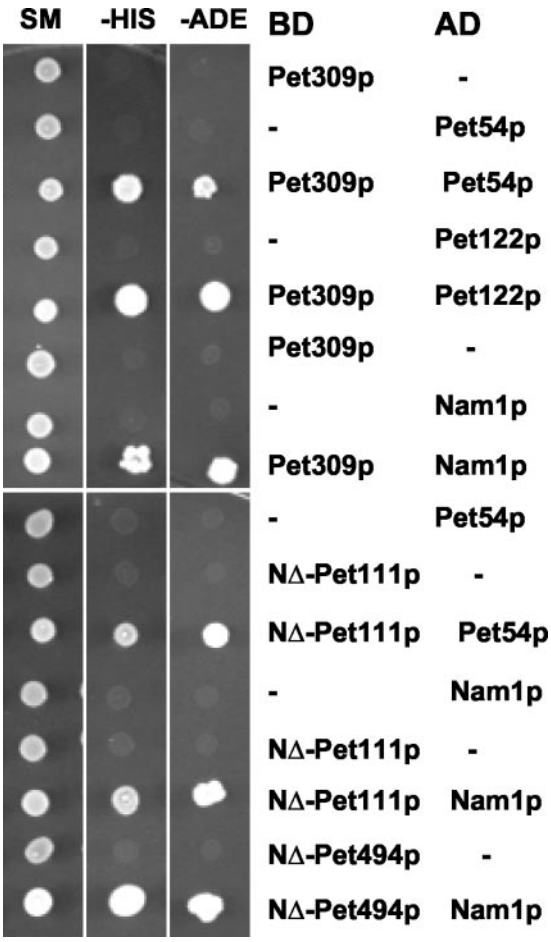

Figure 4. Identification of interactions by use of the yeast two-hybrid system. Plasmid pairs were transformed into host strain PJ69-4a and double transformants were selected on appropriate minimal media. All plasmids encoding transcriptional activation domain (AD) of Gal4p carried *LEU2* selection marker, and vectors encoding DNA binding domain (BD) of Gal4p carried either *TRP1* (pGBT9-based plasmids) or *URA3* (pGBDU-C1) (see MATERIALS AND METHODS). Transformants were grown in liquid minimal medium at 30°C. Then 3 μl of each culture (0.2 OD_{600}), was spotted on plates containing medium selecting for plasmid markers (SM), the *HIS3* reporter (−His) (SC-histidine + 2.5 mM 3-aminotriazole), and the *ADE2* reporter (−Ade) (SC-adenine). Plates were incubated at 30°C for 4 d. The symbol (−) indicates empty vector used in control experiment. In each case, empty vector corresponded to the plasmid carrying the experimental hybrid protein (see MATERIALS AND METHODS).

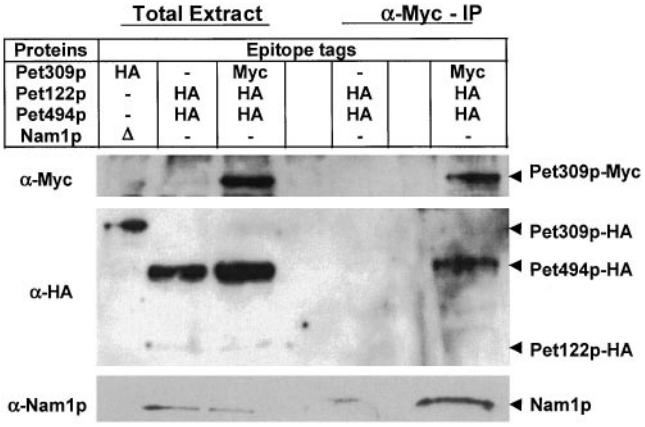

Figure 5. *COX3* activator protein Pet494p, and Nam1p coimmunoprecipitate with the *COX1* activator Pet309p. Purified mitochondria from a strain containing Pet122p-HA, Pet494p-HA, and Pet309p-Myc (SN32), and a strain containing Pet122p-HA and Pet494p-HA (CAB30) were incubated with the cross-linker 2 mM DTME at 25°C for 1 h, before solubilization with 1% digitonin at 4°C for 30 min (see MATERIALS AND METHODS). Clarified mitochondrial extracts (from 500 μg of mitochondria in each case) were incubated with anti-Myc-agarose conjugate at 4°C for 4 h (see MATERIALS AND METHODS). Immunoprecipitates were analyzed by Western blotting with anti-Nam1p, anti-HA, and anti-Myc antibodies. Solubilized proteins from 50 μg of mitochondria were loaded in total extract lane. To establish the identity of Nam1p, 50 μg of total mitochondrial protein from a *nam1*Δ strain, which also contains Pet309p-HA (SN24), was probed.

the *HIS3* reporter weakly (our unpublished data). However, Pet111p did not exhibit two-hybrid interactions with Pet122p or Pet309p (our unpublished data). A fusion protein containing full-length Pet111p fused either to the activating or DNA binding domains of Gal4p failed to exhibit any interactions, including self-interactions.

Coimmune precipitation experiments failed to confirm the interaction between Pet111p and Pet54p, but did confirm the interaction between Pet111p and Pet494p. Purified mitochondria from a strain (SN33) expressing Pet122p-HA, Pet494p-HA, and Pet111p-Myc from their chromosomal loci were treated with DTME, followed by solubilization with 0.5% Triton X-100. The solubilized extracts were incubated with anti-Myc-agarose conjugate and the immunoprecipitates were analyzed by Western blots. More than 10% of the Pet111p-Myc was precipitated from these extracts, whereas <0.5% of Pet494p-HA was coimmunoprecipitated (Figure 6). Significantly less Pet494p was present in the control precipitates from mitochondrial extracts containing unmodified Pet111p. In absence of the cross-linker, the yield of the Pet494p-HA in the coimmunoprecipitates was three- to five-fold lower. Coimmune precipitation of Pet54p and Pet122p-HA with Pet111p-Myc was not observed, nor were any interactions observed between the *COX2* activator Pet111p and the *COX1* activator Pet309p in the two-hybrid system or in coimmune precipitation experiments (our unpublished data).

Nam1p Interacts with Translational Activators Pet309p, Pet111p, and Pet494p

Nam1p/Mtf2p is a soluble matrix protein required for normal mitochondrial mRNA metabolism (Lisowsky and Michaelis, 1989; Wallis *et al.*, 1994). Nam1p has been proposed to facilitate movement of mitochondrial transcripts to their sites of translation on the inner membrane (Wallis *et al.*, 1994; Bryan *et al.*, 2002) and interacts with the mitochondrial RNA polymerase (Rodeheffer *et al.*, 2001). We therefore tested whether Nam1p interacts with translational activator proteins. First, two-hybrid interactions between Nam1p and

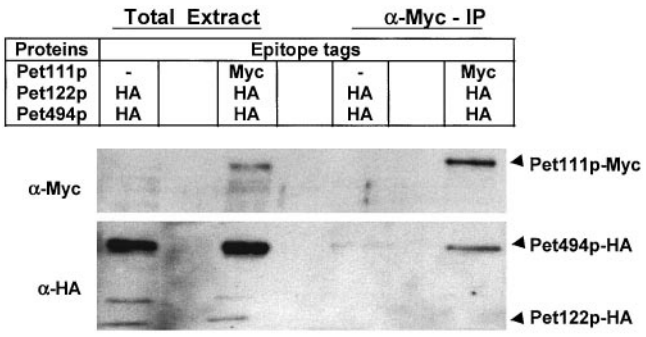

Figure 6. *COX3* activator Pet494p coimmunoprecipitates with the *COX2* activator protein Pet111p. Purified mitochondria containing the indicated epitope-tagged proteins were treated with the cross-linker 2 mM DTME at 25°C for 1 h, followed by solubilization with 0.5% Triton X-100 at 4°C for 30 min. The clarified extracts (from 350 μg of mitochondria in each case) were incubated with the anti-Myc-agarose conjugate for 4 h at 4°C under gentle shaking conditions and immunoprecipitates were analyzed by Western blotting (see MATERIALS AND METHODS). Anti-HA-HRP and anti-Myc-HRP antibodies were used for probing the Western blots. Solubilized proteins from 20 μg of mitochondria were loaded in total extract lanes. Strains were CAB30 and SN33.

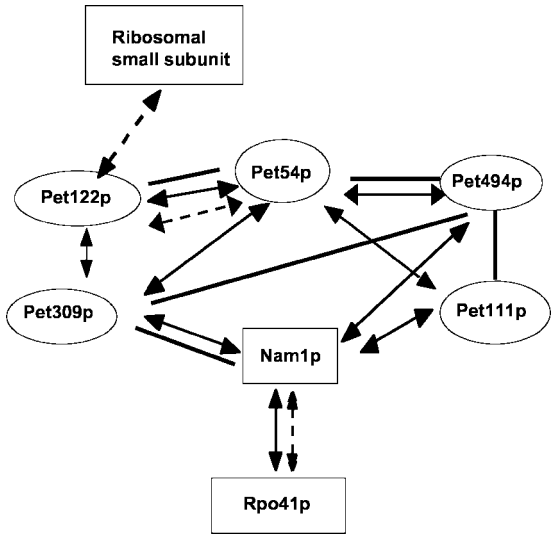

Figure 7. Summary of interactions among proteins involved in mitochondrial gene expression. Thick bars indicate coimmunoprecipitation, double-headed arrows connected by solid lines indicate two-hybrid interactions, and double-headed arrows connected by broken lines indicate genetic/functional interaction. Functional interactions between mitochondrial small subunit ribosomal proteins and Pet122p (McMullin *et al.*, 1990; Haffter *et al.*, 1991; Haffter and Fox, 1992) and interactions between of Nam1p and Rpo41p (mitochondrial RNA polymerase) (Rodeheffer *et al.*, 2001) were reported previously. Two-hybrid and genetic interactions among Pet54p, Pet122p, and Pet494p were reported previously (Brown *et al.*, 1994; Wiesenberger *et al.*, 1995).

the *COX1*, the *COX2*, and the *COX3* activators were examined in pairwise manner (Figure 4). Cells expressing Nam1p-Gal4AD (from pACT-NAM1) or Pet309p-Gal4BD (from pSN21) alone were unable grow in absence of histidine and adenine. However, cells expressing Pet309p-Gal4BD and Nam1p-Gal4AD simultaneously were able to confer histidine and adenine prototrophy, indicating interaction between Nam1p and Pet309p. Nam1p exhibited clear two-hybrid interactions with Pet309p and with the N-terminally truncated forms of Pet111p and Pet494p (Figure 4). No interactions were observed between Nam1p and either Pet54p or Pet122p (our unpublished data).

To test for coimmune precipitation of Nam1p with translational activators, we used an anti-Nam1p polyclonal serum (Wallis *et al.*, 1994) to probe Western blots. This serum recognized a 51-kDa protein in extracts of wild-type mitochondria that was absent in mitochondrial extracts from a *nam1* deletion strain (Figure 5). Coimmunoprecipitation of Nam1p was observed with the *COX1* translational activator Pet309p-Myc (Figure 5), but not with the other translational activator proteins tested.

DISCUSSION

Our results demonstrate associations among nuclearly encoded mRNA-specific translational activator proteins, located on the matrix side of the inner membrane, that control the synthesis of the mitochondrially coded core subunits of *S. cerevisiae* cytochrome *c* oxidase. These interactions, as well as others established previously, are summarized in Figure 7. Because untranslated regions of the mitochondrial *COX2* and *COX3* mRNAs, which contain the targets of their translational activators, play a role in targeting translation for efficient cytochrome *c* oxidase assembly (Sanchirico *et al.*, 1998), our present findings suggest that these interacting activators could be organized on the surface of the inner membrane such that synthesis of Cox1p, Cox2p, and Cox3p would be colocalized in a way that facilitates assembly of the enzymatic core of the cytochrome *c* oxidase complex.

Our data do not distinguish whether interactions among the translational activators are transient and dynamic, or strong enough to form stable complexes in vivo. We were not able to detect stable complexes in detergent-solubilized extracts by using blue native gel electrophoresis (Schägger, 1995; our unpublished data). In any event, their physical association with each other is not required for their activity, because deletion of genes coding for one of the activators does not disrupt the mRNA-specific functions of the others (Fox, 1996a).

Why translation of the mitochondrially coded *COX1*, *COX2*, and *COX3* mRNAs should be dependent on distinct activators, as opposed to a single "cytochrome *c* oxidase-specific activator" remains an open question. One possible rationalization is that mRNA specificity prevents competition among the three mRNAs for a single activator protein, where small differences in affinities could produce large differences in relative rates of synthesis. Interestingly, the amino acid sequences of translational activator proteins have diverged rapidly during fungal evolution, whereas the mRNA-specific relationships between activator protein homologues and target mRNAs have been conserved in those cases studied (Coffin *et al.*, 1997; Costanzo *et al.*, 2000). These orthologous functional relationships have been conserved despite the fact that the activator dependence of mitochondrial mRNAs can be altered experimentally in *S. cerevisiae*,

without eliminating respiratory complex formation, by in vivo expression of chimeric mRNAs containing 5′-UTLs and coding sequences derived from different respiratory complex genes (Müller *et al.*, 1984; Costanzo and Fox, 1986, 1988; Poutre and Fox, 1987; Rödel and Fox, 1987; Mulero and Fox, 1993b; Manthey and McEwen, 1995). Thus, although the interactions among translational activators for the core cytochrome *c* oxidase subunits are likely to confer a selective advantage, they are not absolutely required to form the enzyme.

PET494 expression levels (Marykwas and Fox, 1989) suggest that a diploid cell growing on nonfermentable carbon sources contains roughly 50–100 *COX3*-specific translational activators (Fox, 1996b). The expression of *PET122* and *PET111* seems to be comparably low (our unpublished data). These facts, taken together with the interactions reported herein suggest the possibility that there are a limited number of foci on the inner membrane where synthesis of the core subunits of cytochrome *c* oxidase is initiated. Interestingly, a genetic interaction between *PET111* and *COX18,* which is required to translocate the Cox2p C-tail through the inner membrane, suggests that the levels of the *COX2* translational activator and a Cox2p translocator are roughly comparable (Saracco and Fox, 2002). How the cytoplasmically synthesized subunits of cytochrome *c* oxidase would be targeted to such foci is an interesting question in view of the fact that published data (Vestweber and Schatz, 1988) suggest that there must be at least 10,000 sites per cell where cytoplasmically synthesized precursor proteins can be imported into mitochondria.

Interesting parallels can be drawn between the mRNA-specific translational activators of yeast mitochondria and bacterial type III secretion chaperones. These chaperones are typically specific for a single substrate and in at least some cases seem to function as translational activators with a role in targeting translation to the translocation apparatus, at least for flagellar assembly (Karlinsey *et al.*, 2000; Aldridge and Hughes, 2001). Although there is no detectable homology between the mitochondrial proteins and any type III secretion components, the mechanisms used in yeast mitochondria are likely to have their origins in bacterial systems.

A substantial fraction of mammalian mitochondrial ribosomes are tightly associated with the inner membrane (Liu and Spremulli, 2000). However, it remains unknown whether or how translation of mammalian mitochondrial mRNAs is localized on the inner membrane. A mechanism to achieve this in animals must be at least somewhat different from that of yeast because animal mitochondrial mRNAs lack 5′-UTLs (Attardi and Schatz, 1988), which contain the recognition sites for yeast activators. An mRNA-specific mammalian system analogous or homologous to that of yeast would have to recognize RNA sites embedded in the coding sequences.

The interactions we observed between Nam1p/Mtf2p and translational activators suggest a direct link between transcription and translation within the mitochondrial matrix (Figure 7). Nam1p is a soluble matrix protein (Wallis *et al.*, 1994) encoded by a gene originally identified as a high-copy suppressor of mitochondrial intron splicing defects and by temperature-sensitive mutations affecting mRNA levels (Lisowsky and Michaelis, 1989; Wallis *et al.*, 1994). Nam1p is required to stabilize intron-containing mRNAs and has been proposed to "convey" mitochondrially coded mRNAs to the translation apparatus (Wallis *et al.*, 1994) in a pathway involving the inner membrane protein Sls1p (Bryan *et al.*, 2002). Importantly, Nam1p was recently found to interact with the amino terminal domain of the core subunit of mitochondrial RNA polymerase, Rpo41p, both genetically and in a two-hybrid test (Rodeheffer *et al.*, 2001). Taken together, these data suggest the interesting possibility that mitochondrial transcriptional machinery is coupled to the membrane-bound translational activation system.

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