Cox11 is an intrinsic mitochondrial membrane protein essential for the assembly of an active cytochrome c oxidase complex. Cox11 is tethered to the mitochondrial inner membrane by a single transmembrane helix. Domain mapping was carried to determine the functional segments of the Cox11 protein. The C-terminal 189 residue Cu(I) binding domain is shown to be exposed within the mitochondrial intermembrane space (IMS). This orientation was demonstrated by the proteolytic susceptibility of a C-terminal Myc epitope tag in mitoplasts but not in intact mitochondria. Fusion of the N-terminus of Cox11 to a matrix ribosomal protein Rsm22 results in a functional protein capable of suppressing the respiratory defect of both ∆∆∆∆cox11 and ∆∆∆∆rsm22 cells. The functionality of the fusion protein suggests that the Cox11 N-terminus projects into the matrix. The fusion of the C-terminal segment of Cox11 to Rsm22 resembles a naturally occurring fusion of Cox11 in Schizosaccharomyces pombe to a sequence homologous to Saccharomyces cerevisiae Rsm22. Studies on a series of SCO1/COX11 chimeras reveal that the matrix domain of Cox11 lacks a specific function, whereas the Cu(I) binding/donating function requires the yeast Cox11 sequence. The Cu(I) binding domain from human Cox11 cannot functionally replace the yeast sequence. The Cu domain of Cox11 may be an important docking motif for Cox1 or a Cox1 associated protein.

Cytochrome c oxidase (CcO) is the terminal enzyme in the respiratory chain of eukaryotes. Electrons generated from the oxidation of nutrients are channeled through the respiratory chain within the mitochondrial to CeO, where molecular oxygen is reduced to water (1). Mammalian CeO consists of a 13 subunit complex embedded within the mitochondrial inner membrane (IM). The IM differs from the outer membrane in being highly convoluted, folding into tubular structures called cristae where respiratory complexes predominate. The core enzyme, homologous to prokaryotic CeO, consists of three subunits containing the necessary cofactors for the reduction of oxygen and proton pumping (2,3). The three core subunits, Cox1-3, are encoded by the mitochondrial genome, whereas the remaining ten subunits are small nuclear-encoded peptides that pack on the outside of the core complex (3). The peripheral subunits are believed to be important in regulation of the CeO function (4).

The assembly of functional CeO is a multistep process. Subunits are synthesized on cytosolic and mitochondrial ribosomes, so coordinate recruitment of the various subunits to an assembly site must occur (5). CeO requires multiple cofactors. Two heme A moieties exist within Cox1. Copper ions exist in two centers designated CuA and CuB enfolded by Cox2 and Cox1, respectively. The CuA center in Cox2 is the site of initial reduction by cytochrome c (6). The CuB center is buried within Cox1 forming a binuclear site with one of the two heme A cofactors (heme A3). Mg(II) and Zn(II) ions are also inserted in Cox1 and Cox4, respectively.

As the focus of the present study is on formation of the CuB center in Cox1, the assembly of Cox1 will be reviewed. COX1 translation occurs on mitochondrial ribosomes associated with the IM. The ribosomal association with the IM is mediated in part by recruitment of the COX1 mRNA to the IM by Pet309 and translation is dependent on Mss51 and Shy1 (7-9). Two hemes A and single copper and magnesium ions are inserted into Cox1 during the assembly process. The heme A cofactor is synthesized from heme B by the addition of a hydroxethylfarnesyl group by the Cox10 farnesyl transferase and oxidation of a pyrrole methyl group to a formyl group in reactions requiring Yah1 ferredoxin and Arh1 ferredoxin reductase.
The two heme A cofactors are deeply buried within Cox1 suggesting that heme A insertion must occur at an early step in assembly of CcO. Consistent with this prediction, a *Rhodobacter sphaeroides* mutants lacking Cox2 and Cox3 contained a single heme A moiety in the free Cox1 subunit (13).

Cu$_B$ site formation is dependent on the Cox11 protein. The critical observation suggesting a role for Cox11 in Cu$_B$ site formation was the demonstration that cytochrome c oxidase isolated from *R. sphaeroides* ∆cox11 cells lacked Cu$_B$ but contained both hemes; however, heme A$_3$ showed an altered environment by EPR spectroscopy (14). The magnesium content of CcO isolated from ∆cox11 mutant was also low. *S. cerevisiae* lacking Cox11 have impaired cytochrome c oxidase activity and fail to assemble the holoenzyme, so delineation of the precise step defective in this mutant is not possible (15). The evidence from *R. sphaeroides* suggests clearly that Cox11 is specific for Cu$_B$ site formation and perhaps Mg(II) insertion without affecting binding of other cofactors.

*S. cerevisiae* Cox11 is an intrinsic membrane protein within the inner mitochondrial membrane (15). The protein is predicted to contain a single transmembrane helix (residues 85-107) downstream of the N-terminal mitochondrial targeting sequence (residues 1-45). The C-terminal domain of 189 residues is predicted to be exposed within the soluble mitochondrial inner membrane space (IMS) and binds a single Cu(I) ion (16). Mammalian Cox11 was recently shown to project into the IMS (17). Three conserved Cys residues are ligands for the Cu(I) ion in yeast Cox11 (16). Mutation of any of these Cys residues reduces Cu(I) binding and confers respiratory incompetence and reduced CcO activity. Thus, the residues important for Cu(I)-binding correlate with *in vivo* function, suggesting that Cu(I)-binding is important in Cox11 function. This is confirmed by the recently reported structure of the soluble domain of a Cox11 homolog from *Sinorhizobium meliloti*. The protein adopts an immunoglobulin-like fold and dimerizes upon Cu(I) binding to form a binuclear Cu(I) thiolate cluster at the dimer interface (18).

The steps in CcO assembly have been studied in mammalian cells using either inhibition of protein synthesis or mutant cell lines to trap assembly intermediates. Inhibition of protein synthesis in cultured human cells resulted in the appearance of complexes containing a subset of CcO subunits (19). One subcomplex contained Cox1 and Cox4. Additional support for assembly intermediates comes from cells of patients with mutations in CcO assembly factors. Cells from patients with mutations in *SURF1* or *SCO1* reveal a subcomplex of Cox1 associated with Cox4 and Cox5A (17,20). This subcomplex is not observed in cells from patients with mutations in *COX10* suggesting that heme A insertion may occur prior to formation of the Cox1, Cox4, Cox5A subcomplex. Heme A$_3$ site assembly was recently shown to require SURF1 (Shy1) (21). Mutations have not been observed in human *COX11*, so tools are not available to determine whether Cu$_B$ site formation occurs prior to formation of the Cox1, Cox4, Cox5A subcomplex. However, since the Cu$_B$ ion is buried and in a binuclear complex with heme A$_3$, it is reasonable to assume that Cu$_B$ formation is concurrent with heme A insertion.

One clue as to the timing of Cu$_B$ formation emerged from the observation that Cox11 from *Schizosaccharomyces pombe* exists as a fusion protein with the partner protein being homologous to the *S. cerevisiae* protein Rsm22. Rsm22 was recently found associated with a number of proteins of the small mitochondrial ribosome (22). Consistent with a role of Rsm22 in mitochondrial translation, cells lacking Rsm22 are respiratory deficient. The presence of the Rsm22-like domain at the N-terminus of the *S. pombe* Cox11 fusion protein suggests that Cox11 may function to form the Cu$_B$ site in nascent Cox1 chains emerging from the mitochondrial ribosomes. The interrelationship of Cox11 and Rsm22 is explored in this present study.

**MATERIALS AND METHODS**

*Yeast strains:* Δcox11 yeast cells were obtained from Research Genetics and were in the BY4742 background (mat a, Δleu2 Δlys Δhis3 Δura3). In order to prevent loss of mitochondrial function, the Δrsm22 strain was generated with a plasmid-born functional copy.
of RSM22 fused to COXI1. The \( \Delta rsm22 \) strain was generated by first transforming W303 with pRSM22/COXI1, then integration of the KanMX cassette flanked by 350 bp of 5’ and 3’ of the untranslated region of RSM22. Integration into the desired RSM22 locus was verified by PCR and sequencing of genomic DNA of positive transformants. Analysis of the null strain and RSM22 was permitted by transformation with the desired constructs on a HIS3-selectable vector followed by selection on FOA plates. Strain LMA87, bearing TAP-selectable vector followed by selection on HIS3 transformation with the desired constructs on a Integration into the desired of the untranslated region of KanMX cassette flanked by 350 bp of 5 and 3’ DNA of positive transformants. Analysis of the affect its ability to complement the mutation which in the context of site. The restriction site created a R->K containing codon for T84, followed by the sequence of the stop codon was amplified with primers containing a XhoI site was engineered 5 to the stop codon of RSM22 in pHc6, and fused to codons for L61 and T62 in COXI1. Positive transformants were sequenced to verify there were no mutations, and subcloned into pRS426 in separate steps to generate pHc7 with HIS3 selection. The fusion product was also cloned into pRS423 (URA3 selection) for plasmid shuffle experiments.

**Plasmids:** Two SCO1/COXI1 chimeras were engineered. The matrix domain of Cox11, containing residues 55-75, was replaced with the corresponding segment of Sco1, residues 48-68. The resulting molecule contained the mitochondrial target sequence of Cox11, the matrix domain of Sco1 and Cox11 sequences downstream of Sco1 residue 68. Overlapping PCR was used to generate the COXI1/SCO1/COXI1 chimera, containing a 2x Myc epitope tag at the 3’ end of the ORF, which was inserted into the YCp pRS416 with the MET25 promoter and CYC1 terminator. The 5’ 98 codons of SCO1 were fused in frame to codon 100 of yeast COXI1 through a HindIII site. The restriction site created a R->K mutation which in the context of SCO1 did not affect its ability to complement the \( \Delta sco1 \) phenotype. Yeast/human COXI1 chimeras were engineered in vector pRS316 with the MET25 promoter cloned into the SacI-XbaI sites and the CYC1 terminator cloned into the XhoI-KpnI sites. Yeast COXI1 from the ATG to 75 bp 3’ of the stop codon was amplified with primers containing XbaI and HindIII sites, respectively, and inserted into pRS416 (pHC10). Template for human COXI1 was obtained from Research Genetics. Clone 5224659 contained a complete contig that aligned with the predicted sequence of human COXI1. This construct was amplified with primers containing XbaI and HindIII sites and ligated into pRS416 under the control of the MET25 promoter and CYC1 terminator (vector pHc11). pHc12 contains the sequence coding for a chimera containing the yeast COXI1 up to the codon for T84, followed by the sequence coding for the human COXI1 from the codon for T95 to the stop codon. pHc13 contains the sequence coding for a chimera containing the human COXI1 up to codon S110 followed by the yeast COXI1 from the codon for Y101 to the stop codon. Myc-tagged epitopes were generated by overlap extension PCR, inserting two Myc epitopes 5’ to the stop codons. Human COXI7 was amplified and subcloned into Ycplac111 under the control of the MET25 promoter and CYC1 terminator.

Vector encoded RSM22 was constructed by using a forward primer containing a XhoI site and 325 bp upstream of the start codon of RSM22 and a reverse primer containing an HindIII site and 300 bp 3’ to the stop codon. The expected 2.5 kb fragment was cloned into pRS426 (pHC6) and sequenced. The fusion RSM22-COX11 was generated by mutagenesis: a SpeI site was engineered 5’ to the stop codon of RSM22 in pHc6, and fused to codons for L61 and T62 in COXI1. Positive transformants were sequenced to verify there were no mutations, and subcloned into pRS426 in separate steps to generate pHc7 with HIS3 selection. The fusion product was also cloned into pRS423 (URA3 selection) for plasmid shuffle experiments.

**General methods.** Yeast were grown in standard synthetic media lacking appropriate amino acids for selection of positive clones. Mitochondrial isolations and CcO assays were performed as previously described (23), with the cells grown with galactose as the carbon source. Serial dilution drop tests were plated on glucose and glycerol as usual. PCR was performed using Taq or Pfu, depending on length of construct, followed by sequencing to verify the absence of undesirable mutations. Westerns were developed using the following dilutions of antibodies: 1:5000 anti-myc, then 1:10000 anti-mouse HRP, or 1:15k anti-porin, 1:3000 anti-PGK, 1:5000 anti-SOD2, 1:5000 anti-rabbit HRP. Pierce ECL reagents were utilized for chemiluminescent detection. Mitochondrial localization was determined by trypsin digest of intact, hypotonically lysed, and detergent solubized mitochondria. 100-400 mg mitochondria were pelleted and resuspended in spheroplasting buffer (0.6 M sorbitol/50 mM qHepes pH 7.4), 50 mM Hepes, or 0.25% deoxycholate to be 5 mg/mL. 12.5 mg trypsin or buffer was added and the samples incubated at 4°C for 20 min. Proteolysis was halted by addition of phenylmethylsulfonyl fluoride to 20 mM. Samples were centrifuged to separate soluble proteins from intact mitochondria. Pellets were resuspended in HEPES to the same
volume as the supernatant and 6x SDS buffer was added prior to western analysis.

Immunoprecipitations Immunoprecipitations were performed using mitochondria isolated from LMA87 (RSM22-TAP) transformed with pCOX11-Myc. Mitochondria were pelleted and resuspended in cross-linking buffer to a final concentration of 5 mg/mL. The crosslinker dimethyl 3,3 dithiobispropionimidate (DTBP) from Pierce was added to a final concentration of 2 mM and incubated at room temperature for 30 min. Cross-linking was stopped by addition of Tris pH 8 to a final concentration of 150 mM. Mitochondria were pelleted and resuspended in solubilization buffer, and incubated with gentle shaking at 4°C. Insoluble material was separated from soluble proteins by centrifugation at 4°C (16,000 x g for 15 min). Supernatant was cleared by incubation with Sepharose for 30 min. After clarification, the mitochondrial lysates were incubated with rabbit IgG agarose for 4 to 8 h at 4°C. Unbound proteins were washed with 3 volumes of solubilization buffer. IgG beads were boiled in the presence of 100 mM DTT and SDS buffer and Westerns were performed.

RESULTS

Domain mapping was carried out on yeast Cox11 to determine which segment of the polypeptide is essential for its role in the assembly of cytochrome c oxidase. Cox11 contains a single transmembrane (TM) domain that separates a short N-terminal domain and a 22 kDa soluble domain that is predicted to project into the IMS (Fig. 1A).

We initially sought to confirm this membrane orientation of Cox11. Mitochondria and mitoplasts isolated from Δcox11 cells containing the C-terminal Myc-tagged Cox11 were incubated in the presence and absence of trypsin prior to western analysis. The Myc epitope was proteolytically cleaved in mitoplasts and detergent-solubilized mitochondria but not whole mitochondria consistent with the C-terminal segment projecting into the soluble IMS (Fig. 2).

To determine the functional significance of the three domains of Cox11, chimeric molecules were engineered replacing each domain with a corresponding domain from a second CcO assembly protein Sco1 that has a similar domain structure and also resides within the mitochondrial inner membrane (12). Since the C-terminal Cu(I) binding domain projects into the IMS, the N-terminal segment upstream of the TM must exist within the matrix. To test whether this matrix domain is functionally important, the segment encoding the matrix domain of COX11 (codons 55-75) was replaced with the corresponding segment of SCO1 (codons 48-68). Cells (Δcox11) transformed with this fusion gene, designated mSCO1/COX11, were respiratory competent.

Western analysis confirmed the accumulation of the Myc-tagged chimeric protein (Fig. 3B). The functionality of this chimera and the lack of sequence similarity in the matrix domain between yeast and human Cox11 (Fig. 1B) confirm that the matrix domain is nonessential for Cox11 function (Fig. 3A).

The second domain, the transmembrane (TM) domain, of Cox11 shows sequence conservation among eukaryotic species. To determine whether the sequence of this segment is essential for the Cox11 physiological function, a segment of COX11 encoding the 3100 codons was fused to the 598 codons of SCO1 resulting in Sco1/Cox11 chimera containing the mitochondrial target sequence and TM of Sco1 fused to the Cu(I)-binding soluble domain of Cox11. This chimeric gene failed to complement a Δcox11 strain of S. cerevisiae (Fig. 3A), even though the chimera was stably expressed within the mitochondrion (Fig. 3B).

The third domain is the IMS Cu(I) binding domain. As expected, the Cu(I) binding domain of Sco1 cannot replace the Cu(I) binding domain of Cox11. A chimeric protein consisting of residues 1-107 of Cox11 fused to residues 98-295 of Sco1 was nonfunctional (data not shown). As a more conservative approach, we tested whether the human COX11 can complement Δcox11 yeast cells. Transformants of Δcox11 cells with Myc-tagged human COX11 failed to grow on glycerol medium (Fig. 4B), but little Myc-tagged hCox11 protein accumulated within mitochondria (Fig. 4D). Human COX11 failed to complement Δcox11 cells even when overexpressed. Although the Cu(I) binding domains of human and yeast Cox11 are highly similar in sequence, the two proteins differ in that the yeast protein is considerably longer at the C-terminal end (Fig. 1B, 4A). However, the C-terminal extension in yeast Cox11 is
functionally unimportant, as a C-terminal truncate of yeast Cox11 lacking the last 37 residues did not impair function (data not shown).

The non-functionality of human Cox11 in yeast Δcox11 cells may arise from the sequence variation within the Cu-binding domain. To test this prediction, a human/yeast chimeric Cox11 molecule was engineered with the boundary separating human and yeast sequences near the end of the TM domain where the two sequences are identical (residues 1-110 of human Cox11 fused to residue 101 of yeast Cox11) (see arrow in Fig. 1B, Fig. 4A). The chimeric gene was expressed in Δcox11 cells under the control of the yeast COX11 promoter and terminator. Fusion of the human Cox11 N-terminus containing the TM to the yeast Cox11 Cu-binding domain (construct 3 in Fig. 4A) allowed weak glycerol growth (Fig. 4B) and cytochrome c oxidase activity of Δcox11 cells (data not shown). The weak complementation was improved by overexpression of this chimera (data not shown).

To test whether the human Cu(I) binding domain could functionally replace the yeast Cu(I) binding domain, a yeast/human COX11 chimera was constructed with a similar junction to the partially functional human/yeast chimera. The yeast/human chimera (construct 4 in Fig. 4A) was unable to suppress the glycerol auxotrophy of a Δcox11 strain even though the protein was well expressed (Fig. 4B, Fig. 4C). A yeast/human Cox11 chimera was constructed in which the boundary was moved further downstream of the TM (residue 161, chimera 5) (Fig. 4A). This chimera was nonfunctional when expressed in Δcox11 cells under the yeast COX11 promoter and terminator or when overexpressed (data not shown).

The nonfunctional state of human Cox11 Cu(I) binding domain may arise from an inability of yeast Cox17 to deliver Cu(I) to the human Cox11 due to an attenuated interaction between the two proteins. We demonstrated that Cox17 is a physiological Cu(I) donor to Cox11 (26). The addition of human Cox17 to yeast cells containing the yeast/human Cox11 chimera (construct 4 in Fig. 4A) failed to restore glycerol growth to Δcox11 cells containing human Cox11 Cu-binding domain (data not shown). These cells contained a wild-type yeast Cox17 that would be competent to transfer Cu(I) to Sco1. Suppression was observed only when yeast COX11 was transformed into these cells. It was not possible to test the prediction that human Cox11 requires a human Cox1 target as COX1 is mitochondrially encoded and translational activators for human COX1 are not identified.

The Cox11 homolog in S. pombe contains a 520 amino acid N-terminal extension (Fig. 5A). The extension sequence is highly similar to the S. cerevisiae protein Rsm22, which is associated with the mitochondrial small ribosomal subunit (22). The fusion is the only copy of the Rsm22-like sequence in S. pombe. To test whether a fusion of S. cerevisiae Rsm22 and Cox11 proteins can functionally replace each individual molecule, a chimeric protein was engineered such that the RSM22 ORF was fused in frame to codon 61 of COX11. Removal of the first 60 codons of COX11 removes the mitochondrial targeting sequence (Fig. 1A). Thus, cleavage of the fusion protein prior to mitochondrial uptake would result in a failure of the Cox11 fragment to be properly imported into the mitochondrion. Cells lacking Cox11 (Δcox11) were transformed with an episomal RSM22/COX11 fusion gene and plated on medium containing the non-fermentable carbon source glycerol. Whereas Δcox11 cells fail to grow on glycerol-containing medium, Δcox11 transformants with pRSM22/COX11 grew as well as wild-type cells (Fig. 5B). Western analysis of cells containing the Myc-tagged Rsm22/Cox11 fusion revealed a prominent band of the expected size for the chimeric protein (Fig. 5C). Quantitation of cytochrome c oxidase activity of purified mitochondria isolated from Δcox11 transformants with pRSM22/COX11 revealed greater than wild-type levels of activity (Fig. 5D). The functionality of the Rsm22/Cox11 fusion confirms that the N-terminus of Cox11 projects into the matrix.

Rsm22 is important for maintenance of mitochondrial DNA. Since cells lacking Rsm22 are petite, RSM22 was deleted in cells harboring a URA3-based vector encoding RSM22. Cells containing a disruption of the chromosomal RSM22 locus were transformed with the pRSM22/COX11 fusion gene on a HIS3-based vector prior to shedding the URA3-based RSM22 vector using 5-FOA. Cells (Δrsm22) containing only the pRSM22/COX11 plasmid were able to propagate on glycerol-containing medium (Fig. 5B). Mitochondria isolated from the
transformants showed wild-type levels of cytochrome c oxidase activity suggesting that the fusion protein was fully functional (Fig. 5D).

The functionality of the fusion protein is consistent with a potential interaction in S. cerevisiae. To test for an interaction between the two proteins, cells containing a TAP-tagged chromosomal RSM22 locus was transformed with a MYC-tagged COX11 fusion. The TAP tag encodes a calmodulin-binding peptide followed by protein A (24). Mitochondria isolated from the transformants were detergent solubilized and precipitated with immunoglobulin beads either with or without crosslinking. Immunoprecipitation of TAP-Rsm22 was successful and multiple proteins were copurified, however western analysis of the immunoprecipitate with anti-Myc antibodies did not reproducibly show co-immunoprecipitation of Cox11 and Rsm22. Thus, any potential interaction of endogenous Rsm22 and Cox11 may be only transient or indirect in S. cerevisiae.

DISCUSSION

Cox11 is an intrinsic membrane protein with a single TM domain tethering the protein within the IM (15). The 189 residue C-terminal Cu(I) binding domain was predicted to be exposed within the IMS (16), and this orientation was confirmed by two independent approaches in the present study. First, insertion of a myc-epitope tag at the C-terminus of Cox11 resulted in proteolytic susceptibility in mitoplasts but not intact mitochondria. Second, the fusion of Cox11 with a candidate matrix ribosomal protein in S. pombe is consistent with the Cox11 N-terminus being within the matrix. The corresponding partner protein in S. cerevisiae is the mitochondrial matrix protein Rsm22. A fusion of Rsm22 to the N-terminus of Cox11 resulted in a stable fusion protein that was able to suppress the respiratory defect of both Δcox11 and Δrsm22 cells. The N-terminus of Cox11 must project into the matrix resulting in the Cu(I)-binding domain of Cox11 projecting into the IMS.

Studies of human/yeast Cox11 chimeric molecules revealed the importance of the yeast Cu(I) binding domain sequence for function within S. cerevisiae. Replacing the yeast Cu(I) binding domain by the human equivalent failed to generate a functional complex even though the chimeric protein was stable. Likewise, the Cu(I) binding domains of human Sco1 and Sco2 fail to functionally replace the yeast domain (25). However, a segment of human Sco1 downstream of the CxxxC motif can replace the corresponding segment of yeast Sco1 (25). A corresponding yeast/human chimera of Cox11 is nonfunctional. In the case of Cox11, sequence differences in the human Cu domain may attenuate an important interaction for a Cu(I) donor protein or a Cox11-associated protein. The inability of human COX11 to complement yeast Δcox11 cells is not reversed by human COX17 that is weakly functional in yeast (27), suggesting that human Cox11 may be impaired in its interaction with another protein, perhaps Cox1 itself.

The fusion of Cox11 with a candidate matrix ribosomal protein in S. pombe suggests that Cox11 function may occur during mitochondrial translation of the COX1 mRNA. COXI translation is known to occur on ribosomes tethered to the inner membrane by an interaction with the inner membrane protein Pet309 that binds to a structural element in the 5 UTR of COX1 (7). Pet309 is associated with the other translational activators for COX1 and COX2 (28). One of the COX3 activators, Pet122, appears to directly interact with a ribosomal protein in the small subunit (29). Thus, translation of COX1, COX2 and COX3 is tightly associated with the IM. It is likely that translation of COX4 is coupled with the insertion of heme A and Cu cofactors, since the cofactors are buried and a candidate channel for Cu addition in Cox1 is blocked when Cox1 associates with Cox2. Recently, Shy1 was shown to be important for heme A3 site formation (21). Heme A3 and CuB site formation can occur independently of each other (14, 21). Thus, recruitment of both Shy1 and Cox11 to sites on the IM involved in COX1 translation is likely.

The recruitment of Cox11 to sites of COX1 translation may be mediated by an interaction with Rsm22. We were unsuccessful in detecting any stable interaction between Cox11 and Rsm22. If an interaction exists, it is unlikely that the interaction interface consists of the matrix domain of Cox11 as a Sco1/Cox11 chimera containing the Sco1 matrix domain in place of the Cox11 matrix domain was fully functional and the human Cox11 matrix
sequence is not conserved with the yeast sequence. Upon cleavage of the mitochondrial target sequence in yeast Cox11, only 39 residues protrude into the matrix.

While this paper was under review, a report appeared showing a weak interaction of Cox11 with the mitochondrial ribosomal protein MrpL36 (30). Khalimonchuk et al. showed that Cox11 fractionated with mitoribosomes and suggested that CuB formation in Cox1 occurs cotranslationally (30). Our data on domain mapping suggest that any interaction of Cox11 with a ribosomal protein would be indirect, as the matrix domain is non-essential. An interaction between Rsm22 and Cox11 may be mediated by another protein, such as Pet309. Pet309 has multiple TM domains, so Cox11 may also be recruited to the site of Cox1 synthesis by interaction with IMS or TM segments of Pet309. Ribosomal proteins are abundant molecules, unlike Cox11, so it is curious that the S. pombe homolog is fused to a ribosomal protein. However, neither Rsm22 nor Cox11 appear to be stoichiometric with many S. cerevisiae ribosomal subunit proteins (22). Neither Rsm22 nor Pet309 have obvious homologs outside fungi, so it is unclear whether similar assembly steps occur in mammalian cells.

Acknowledgements
This work was supported by a grant ES 03817 from the National Institutes of Environmental Health Sciences, NIH to D.R.W.

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29. FIGURE LEGENDS

Figure 1. Yeast Cox11 domain structure. The domains of yeast Cox11 we define in this study are shown in (A). The alignment of human (H) and S. cerevisiae (Y) Cox11 proteins is shown in (B) with the matrix and TM domains highlighted. The sequence for human Cox11 starts at the putative mature protein after cleavage of the mitochondrial import sequence as predicted by PSORT. The mature yeast Cox11 starts following the slash with only a segment of the upstream mitochondrial import sequence shown. The arrow shows the site of the boundary for human/yeast chimeras.

Figure 2. Trypsin susceptibility of the C-terminal Myc tag on Cox11. 100 µg mitochondria were treated as described in the experimental section to generate intact mitochondria, mitoplasts (MP), and DOC solubilized mitochondria. After treatment with trypsin and processing as described, samples were separated on a 12% gel and transferred to nitrocellulose. The membranes were then probed for Myc and for the mitochondrial outer membrane protein porin.

Figure 3. Function of Sco1/Cox11 chimeras. Panel A: Cells (Δcox11) transformed with an empty vector, wild-type yeast COX11, or two SCO1/COX11 different chimeras were plated on agar medium containing either dextrose, glycerol/lactate or glycerol carbon sources. Panel B: Western analysis of Cox11 variants from transformants in panel A. Lanes 1 and 4 are samples from Cox11-Myc cells. Lanes 2 and 5 are samples from TM Sco1/Cox11 cells (Sco1 sequences through TM). This construct does not have a Myc tag. Lanes 3 and 6 are samples from matrix Sco1/Cox11 cells (Sco1 sequence only in matrix domain). Lanes 1-3 are mitochondrial fractions, lanes 4-6 are post-mitochondrial supernatants. Cox11 was visualized using either antisera to Cox11 or anti-Myc for Myc-tagged chimeras.

Figure 4. Complementation of Δcox11 cells by human COX11 and yeast/human COX11 chimeras. A schematic of the domains swapped to generate yeast/human chimeras is depicted in panel A. The boundary for human/yeast and yeast/human chimeras in constructs #3 and #4 is residue Tyr101 in yeast Cox11. The sequence at the end of the TM is identical in both yeast and human Cox11 (YAAVPLY) as seen in Fig. 1B. For growth on variable carbon sources, cells harboring the plasmid indicated were diluted to equal cell density and plated by serial dilutions on selective media containing glucose or glycerol (panel B). Normalized protein expression was assessed by western analysis are shown (panel C).

Figure 5. A fusion protein consisting of Rsm22 and Cox11 is functional. The domain structure of Rsm22 and the fusion constructed are demonstrated in (A). Null strains shown (B) were grown in selective media and diluted to equal cell density before being dropped onto selective media containing the carbon source indicated. The Δrsm22 strain underwent plasmid shuffle to remove the URA3-born copy of RSM22 as described in the experimental section. Expression of Myc-tagged protein is shown in panel C. Mitochondrial lysates from cells (Δcox11) transformed with COX11-Myc (lane 1) or RSM22/COX11-Myc (lane 2) were electrophoresed on SDS-PAGE and subjected to western analysis using anti-Myc antisera. Cytochrome c oxidase activity as a percent of WT (D) is presented in panel D.
Fig. 1A

- Mitochondrial target sequence
- Matrix domain swapped
- TM transmembrane domain
Fig. 1B
Fig. 2

Anti-Myc

Anti-porin

Tp:  

|    | Con | Intact | MP | DOC |
|----|-----|--------|----|-----|
| Tp | -   | -      | +  | -   |
| Tp | -   | -      | +  | +   |
| Tp | -   | +      | +  | +   |
\textit{cox11}Δ transformants:

+ \textit{COX11}
+ vector
+ m \textit{SCO1/COX11}
+ TM \textit{SCO1/COX11}

Dextrose \hspace{1cm} \text{Lactate+Glycerol} \hspace{1cm} \text{Glycerol}

Fig. 3A
| Mito fraction | Post-Mito fraction |
|--------------|-------------------|
| 1            | 4                 |
| 2            | 5                 |
| 3            | 6                 |

- **Mito fraction**: Myc, PGK, Porin
- **Post-Mito fraction**: Cox11, Myc, PGK, Porin

**Fig. 3B**
| Construct:       | MTS | Matrix | TM   | Cu-BD | C-tail |
|------------------|-----|--------|------|-------|--------|
| 1. Human         |     |        |      |       |        |
| 2. Yeast         |     |        |      |       |        |
| 3. Human/yeast   |     |        |      |       |        |
| 4. Yeast/human   |     |        |      |       |        |
| 5. Yeast/human   |     |        |      |       |        |

Fig. 4A
Fig. 4B
Fig. 4C
Sp Cox11 (753 aa)

Sc Cox11 (300 aa)

Sc Rsm22 (628 aa)

Rsm22-Cox11 fusion (869 aa)
Fig. 5B

Glucose

\[ \Delta \text{cox11} \]

- \( pCOX11 \)
- \( pFusion \)
- \( pRSM22 \)
- vector

\[ \Delta \text{rsm22} \]

- \( pRSM22 \)
- pFusion

Glycerol
Fig. 5D
Functional analysis of the domains in Cox11
Heather S. Carr, Andrew B. Maxfield, Yih-Chern Horng and Dennis R. Winge

J. Biol. Chem. published online April 19, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M414077200

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