Deletion of reading frame YHR116W of the Saccharomyces cerevisiae nuclear genome elicits a respiratory deficiency. The encoded product, here named Cox23p, is shown to be required for the expression of cytochrome oxidase. Cox23p is homologous to Cox17p, a water-soluble copper protein previously implicated in the maturation of the Cu₆ center of cytochrome oxidase. The respiratory defect of a cox23 null mutant is rescued by high concentrations of copper in the medium but only when the mutant harbors COX17 on a high copy plasmid. Overexpression of Cox17p by itself is not a sufficient condition to rescue the mutant phenotype. Cox23p, like Cox17p, is detected in the intermembrane space of mitochondria and in the postmitochondrial supernatant fraction, the latter consisting predominantly of cytosolic proteins. Because Cox23p and Cox17p are not part of a complex, the requirement of both for cytochrome oxidase assembly suggests that they function in a common pathway with Cox17p acting downstream of Cox23p.

Copper is an essential electron carrier of cytochrome oxidase, the terminal complex of the respiratory chain. The copper in cytochrome oxidase is associated with two separate catalytic sites (1, 2). Two of the copper forms the Cu₆ center in subunit 2 (Cox2p). The third copper, referred to as Cu₅, is in electronic equilibrium with the heme a of cytochrome c₃ in subunit 1 (Cox1p). The Cu₆ center is located in a carboxyl-terminal domain of Cox2p that protrudes into the intermembrane space of mitochondria where it acts as the initial acceptor of electrons from cytochrome c (1, 2). Acquisition of copper for the Cu₆ center of Cox2p has been shown to depend on at least two proteins (3–5). Cox17p is a small soluble copper protein that is detected in the cytosol and in the mitochondrial intermembrane space (6, 7). This subcellular localization suggested that Cox17p functions in delivery of cytosolic copper to mitochondria (3). The second protein, Sco1p, is a constituent of the inner membrane (8) and like Cox2p has a carboxyl-terminal domain with the copper binding site located in the intermembrane space (8, 9). The function of Sco1p is either to transfer copper from Cox17p to the Cu₆ center of Cox2p or to chemically modify the oxidation state of the cysteine ligands for copper (5, 10).

The Cu₆ center on Cox1p is probably formed in the lipid phase of the inner membrane or in the matrix compartment.

Cox11p, a mitochondrial inner membrane protein previously shown to be essential for the expression of yeast cytochrome oxidase (11), has been implicated in the maturation of the Cu₆ site of Cox1p (12). Mutations in the COX11 homologue of Rhodobacter sphaeroides elicit a cytochrome oxidase deficiency and an absence of Cu₅ but not Cu₆ (12). Additional support for the involvement of Cox11p in this process comes from the finding that yeast Cox11p is a copper protein (13). It is not known whether other proteins may also participate in the maturation of this important catalytic center.

As part of an effort to understand cytochrome oxidase assembly, we have continued to screen for genes that affect the expression of this respiratory complex in yeast. We show that reading frame YHR116W of chromosome VIII (named COX23) elicits a cytochrome oxidase deficiency and is essential for the expression of functional cytochrome oxidase.

MATERIALS AND METHODS

Yeast Strains and Growth Media—The genotypes and sources of the strains of Saccharomyces cerevisiae used in this study are described in Table I. The compositions of the media used for the cultivation of yeast have been described elsewhere (11).

Construction of Genes Expressing Cox17p and Cox23p Tagged with Hemagglutinin or with Hemagglutinin Plus Polyhistidine—Hybrid COX17 and COX23 genes coding for Cox17p-HA and Cox23p-HA with a carboxyl-terminal residues constituting the hemagglutinin (HA) tag were obtained by PCR amplification. COX17 expressing the HA-tagged protein was amplified from pG74/T8 (3) with primers 5’- CATACTATC-TAAGCTTATTCTA and 5’-GGCAAGACCTCGATCATGCGGAC-GGTGCATAATGATTTGCAATCCTGGAACTTCAAAGC. The product was digested with HindIII and cloned in the yeast/E. coli shuttle plasmid Yip351 (15). The resultant plasmid (pG74/ST52) was linearized at the ClaI site in the LEU2 gene of the vector and integrated into the chromosomal DNA of BW3 Cox17,23/ST4. The plasmid (pG74/ST50) expressing Cox17p tagged with HA and polyhistidine was made in the same way except that the downstream primer was 5’-GGCAAGACCTCGATCATGCGGAC-GGTGCATAATGATTTGCAATCCTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC. Similarly COX23 expressing the HA-tagged protein was amplified from nuclear DNA with primers 5’-ATGCCGGATCCCTTGTATTAG and 5’-GGCCGATCC-CGACAATGTCGCTGGAGCATCCTGGGGAAGGATC-TATGGGATAAATTGGCACTTGGAACTTCAAAGC.
sequence with the yeast HIS3 gene. Amplification of pCOX23/ST2 with the bidirectional primers 5'-GGCGAGATTCCTTTAAGGCGCTTCCGTTGAGAG and 5'-GGCGAGATTCGACGCTTCTTCTTCTTTGTGTTCTAGCTG resulted in a clean deletion of COX23. The linear product containing the COX23 flanking regions in pUC19 was digested with BglII and ligated to HIS3 on a 1-kb BamHI fragment of DNA. The cox23::HIS3 null allele was recovered from this plasmid as a 1.8-kb BamHI fragment and was substituted by homologous recombination for the wild type gene in the yeast strain W303-1B.

**Isolation and Assays of Mitochondria**—Respiratory enzymes were assayed with mitochondria prepared by the method of Faye et al. (18) except that zymolyase 20T (ICN Biochemicals Inc., Aurora, OH) instead of Glusulase was used for the conversion of cells to spheroplasts. For localization studies mitochondria with intact outer membranes were prepared and ligation of DNA fragments and for the transformation of cells to spheroplasts. For the wild type gene in the yeast strain W303-1B.

**RESULTS**

**Phenotype of the cox23 Deletion Mutant**—Reading frame YHR116W on chromosome VIII of the yeast nuclear genome has been deleted in the wild type strain BY4741 as part of the Saccharomyces genome deletion project and shown to cause a growth defect on glycerol/ethanol as carbon sources (24). Deletion of YHR116W in the wild type strain W303-1B produces a similar but less pronounced phenotype. In either genetic background, however, the respiration-deficient phenotype is leaky because slow growth of the mutants on the non-fermentable substrates is detected after incubation of BYACOX23 for 4–5 days and of W303ΔCOX23 for 2–3 days at 30 °C (data not shown).

The presence in the mutant of cytochromes b, c₁, and c (but not of cytochromes a and a₂) suggested that the respiratory defect stems from a deficiency of cytochrome oxidase (Fig. 1A). This was confirmed by enzymatic assays, which showed the mutant to have less than 1% of the cytochrome oxidase activity measured in wild type (Table II). Both NADH-cytochrome c reductase and ATPase activities were not significantly different from wild type.

**TABLE I**

| Strain          | Genotype                                                                 | Source     |
|-----------------|--------------------------------------------------------------------------|-----------|
| BY4741          | MATa met1530 his3Δ1 leu2Δ3 uro3Δ0                                         | (14)      |
| W303-1A         | MATa ade2-1 his3-1,15 leu2-3,12 trpl-1 ura3-1                            | (3)       |
| W303-1B         | MATa ade2-1 his3-1,15 leu2-3,12 trpl-1 ura3-1                            |           |
| W303ΔCOX17      | MATa ade2-1 his3-1,15 leu2-3,12 trpl-1 ura3-1 cox17::TRP1                | This study|
| aW303ΔCOX23     | MATa ade2-1 his3-1,15 leu2-3,12 trpl-1 ura3-1 cox23::HIS3                | W303ΔCOX17 × aW303ΔCOX23 |
| W303ΔCOX17/ST50 | MATa ade2-1 his3-1,15 leu2-3,12 trpl-1 ura3-1 cox17::TRP1 leu2::pG74/ST50 | This study|
| BWACOX17,23/ST4 | MATa ade2 his3 leu2 trp ura3 cox17::TRP1 cox23::kanXM4 ura3::pCOX23/ST4 | BYACOX23/ST4 × W303ΔCOX17 |
| BWACOX17,23/ST4 | MATa ade2 his3 leu2 trp ura3 cox17::TRP1 cox23::kanXM4 ura3::pCOX23/ST4 | EUROPAN   |
| BWACOX17,23/ST4,50 | MATa ade2 met15 his3 leu2 ura3 cox17::TRP1 cox23::kanXM4 ura3::pCOX23/ST4 | BYACOX23/ST4 × W303ΔCOX17/ST50 |
| BWACOX17,23/ST4,52 | MATa ade2 his3 leu2 trp-1 ura3 cox17::TRP1 cox23::kanXM4 ura3::pCOX23/ST4 leu2::pG74/ST50 | This study|

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**Fig. 1. Phenotypes of cox23 mutant.** A, mitochondria from the wild type BY4741 and the cox23 null mutant BYACOX23 were extracted at a final protein concentration of 5 mg/ml with deoxycholate to solubilize all the cytochromes (25). Difference spectra of reduced versus oxidized extracts were recorded at room temperature. The positions of the a absorption bands of cytochromes a + a₂, cytochrome b, and cytochromes c + c₁ are indicated. B, mitochondrial translation products labeled with [35S]methionine in cycloheximide-poisoned wild type (BY4741) and cox23 mutant (BYACOX23) cells. Proteins were separated by electrophoresis on a 17% SDS-polyacrylamide gel. The mitochondrial ribosomal protein Var1; cytochrome oxidase subunits 1 (Cox1), 2 (Cox2), and 3 (Cox3); cytochrome b (Cyt.b); and ATPase subunit 6 (Atp6) and 8 plus 9 (Atp8/9) are identified in the left margin. C, Western blot analysis of cytochrome oxidase subunits in wild type (BY4741) and cox23 mutant (BYACOX23) mitochondria. Total mitochondrial proteins (20 μg) were separated on a 12% polyacrylamide gel and then transferred to nitrocellulose, and the cytochrome oxidase subunits were detected with monoclonal antibodies against Cox1 and Cox3 and polyclonal antibodies against Cox2, Cox4, and Cox5.
Most cytochrome oxidase mutants have very low steady-state concentrations of Cox1p, Cox2p, and Cox3p caused by their turnover when assembly of the enzyme is blocked. This is true not only of structural gene mutants but also of strains that fail to assemble a functional enzyme as a result of impaired heme a synthesis (26) or maturation of the CuA and/or Cu B sites (3, 11). Although the nuclear-encoded subunits are also affected in such mutants, the extent of their reduction is much less (3, 11, 26). Analysis of cytochrome oxidase subunits under steady-state conditions and pulse labeling of cells in the presence of cycloheximide indicate that the phenotype of the cox17 mutant is very similar to that of most other oxidase-deficient mutants. The lack of spectrally and enzymatically detectable cytochrome oxidase and the deficit of the mitochondrially synthesized subunits point to cytochrome oxidase as the target of the loss of activity in cox17, cox23, and cox17,23 mutants. The CuA site in Cox2p (3, 4) is implicated in copper binding (27). Cox23p is a hydrophilic protein of 151 amino acids with an estimated molecular mass of 17.3 kDa. Truncation of the amino-terminal domain of yeast Cox23p homologues are present in widely divergent organisms (Fig. 2). The yeast protein, however, has an extra 75-residue-long serine-rich sequence at the amino end that is absent in the homologous proteins. Cox23p is also homologous to Cox17p, a copper protein required for the maturation of the CuA site in Cox2p (3, 4). The four cysteines of Cox23p align with cysteine residues in Cox17p, some of which have been implicated in copper binding (27). Cox23p is a hydrophilic protein of 151 amino acids with an estimated molecular mass of 17.3 kDa. Truncation of the amino-terminal domain of yeast Cox23p does not affect its ability to confer wild type growth of the mutant on non-fermentable carbon sources (Fig. 3).

**Rescue of the cox23 null mutant by exogenous copper.** The wild type and mutant strains were grown on solid YPD and replicated on YEPG without and with the indicated concentrations of copper added. The plates were incubated for 30 h at 30 °C. W303-1A is a respiration-competent haploid strain of yeast. BY4741 (Δcox23) and W303Δcox17 (Δcox17) are respiration-deficient strains with null mutations in COX23 and COX17, respectively. BY4741Δcox23pΔcox17p (Δcox23 + Δcox17) is the cox17 mutant transformed with COX17 on a high copy plasmid. W303Δcox17pΔcox23pΔcox23ST1pΔcox23ST1 (Δcox17 + Δcox23) is the cox17 mutant transformed with COX23 on a high copy plasmid. aW303Δcox23p/Δcox17,23p (Δcox17,23) carries null mutations in both COX17 and COX23.

**Table II**

| Strains | Doubling time on YPEG h | Specific activity | ATPase |
|---------|-------------------------|------------------|--------|
|         |                         | NADH- reductase μmol/min/mg | Cytochrome oxidase | +Oligomycin |
|         |                         | Yeast Cox23p 76 | -  | 0.47 ± 0.02 | 5.45 ± 0.1 |
|         |                         | N. crassa 1 | -  | 0.47 ± 0.02 | 5.14 ± 0.1 |
|         |                         | D. melanogaster 1 | -  | 0.47 ± 0.02 | 5.14 ± 0.1 |
|         |                         | H. sapiens 1 | -  | 0.47 ± 0.02 | 5.14 ± 0.1 |
|         |                         | Yeast Cox17p 1 | -  | 0.47 ± 0.02 | 5.14 ± 0.1 |

**Fig. 2.** Homologues of Cox23p. Alignment of yeast Cox23p with Cox17p and putative fungal, fly, and human homologues. Identities and conserved subregions are shaded in black and gray, respectively. The four conserved cysteines are marked by the asterisks. The amino-terminal 75 residues of yeast Cox23p (data not shown) are missing in the other sequences.

**Fig. 3.** Growth phenotype of the cox23 deletion mutant and various transformants. The figure shows serial dilutions of the respiration-competent strain BY4741, the cox23 mutant BY4COX23, the transformant BY4COX23/ST1 with an integrative plasmid expressing a hemagglutinin-tagged Cox23p, and the transformants BY4COX23/ST6 and ST7 expressing Cox23p that lacks the amino-terminal 75 residues from an integrative and episomal plasmid, respectively. The different strains were grown in liquid YPD (1% yeast extract, 2% peptone, 2% glucose) and spotted on solid YPD and YEPG (1% yeast extract, 2% peptone, 2% ethanol, 3% glycerol). The plates were incubated at 30 °C for 2 days.

**Fig. 4.** Rescue of the cox23 null mutant by exogenous copper. The wild type and mutant strains were grown on solid YPD and replicated on YEPG without and with the indicated concentrations of copper added. The plates were incubated for 30 h at 30 °C. W303-1A is a respiration-competent haploid strain of yeast. BY4COX23 (Δcox23) and W303Δcox17 (Δcox17) are respiration-deficient strains with null mutations in COX23 and COX17, respectively. BY4COX23ST1pCOX23ST1pCOX23ST1 (Δcox23 + Δcox17) is the cox17 mutant transformed with COX17 on a high copy plasmid. W303Δcox17pCOX23ST1pCOX23ST1 (Δcox17 + Δcox23) is the cox17 mutant transformed with COX23 on a high copy plasmid. aW303Δcox23p/Δcox17,23 (Δcox17,23) carries null mutations in both COX17 and COX23.
COX23 Is Required for Cytochrome Oxidase Assembly

**Fig. 5. Localization of Cox23p-HA.** A, mitochondria (MT) and the postmitochondrial supernatant (PMS) fraction were isolated from BYΔCOX23/ST4. Samples of the two fractions corresponding to 40 μg of protein were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with either a monoclonal antibody against the hemagglutinin epitope (Cox23p-HA) or a polyclonal antibody against cytochrome b2 (Cyt. b2). B, the indicated amounts of protein were separated by SDS-PAGE and probed with the monoclonal antibody against the HA tag as described for A. C, BYΔCOX23/ST4 mitochondria were suspended at a protein concentration of 10 mg/ml in 0.6 M sorbitol, 20 mM Hepes, pH 7.4. To prepare mitoplasts (MP) the mitochondrial suspension was diluted with 8 volumes of 20 mM Hepes, pH 7.4. For controls, mitochondria (MT) were diluted with 8 volumes of 0.6 M sorbitol, 20 mM Hepes, pH 7.5. Proteinase K (Prot. K) was added to one-half of each sample at a final concentration of 100 μg/ml and incubated for 60 min on ice. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM, and the mitochondria and mitoplasts were recovered by centrifugation at 100,000 × g.

The pellets were suspended in 0.6 M sorbitol, 20 mM Hepes, pH 7.4, and the proteins were precipitated by addition of 0.1 volume of 50% trichloroacetic acid. Following dissociation in sample buffer (21), mitochondrial and mitoplast proteins (40 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against the HA tag (Cox23p-HA), cytochrome b2 (Cyt. b2), and α-ketoglutarate dehydrogenase (α-KGD).

cox23 mutant transformed with COX17 on a high copy plasmid (Fig. 4). The supplementation of rich glycerol/ethanol medium with 2–10 mM CuSO4 did not promote growth of the cpx mutant even after a week of incubation at 30 °C. Copper, however, was effective in restoring respiratory growth of the cpx mutant transformed with COX17 on a high copy plasmid. Growth of the transformant was evident after 30 h of incubation. No rescue of the cpx mutant overexpressing COX23 was seen under these conditions nor was the cpx mutant rescued by SCO1, SCO2, COX19, and COX11, all of which have been implicated in mitochondrial copper metabolism. Although cpx mutants are rescued by copper (3), they grow on glycerol/ethanol medium at a slower rate and require a longer period of incubation.

**Localisation of Cox23p—BYΔCOX23/ST4 is a respiration-competent strain with a cox23 null allele and a chromosomally integrated COX23-ΔHA fusion gene expressing Cox23p with a carboxyl-terminal hemagglutinin tag.** This strain was used to study the location of Cox23p. An antibody against the HA tag detected a 25-kDa protein in mitochondria and the postmitochondrial supernatant fraction (cytosolic proteins) of BYΔCOX23/ST4 (Fig. 5A). Because this signal is not seen in mitochondria of wild type yeast (data not shown), we conclude that it corresponds to the HA-tagged Cox23p (Cox23p-ΔHA). The apparent mass of Cox23p-ΔHA measured by SDS-PAGE is 7 kDa greater than that predicted by the DNA sequence. This is probably caused by anomalous bonding of SDS. The absence of cytochrome b2, a soluble protein of the intermembrane compartment, in the postmitochondrial supernatant makes it unlikely that the Cox23p-ΔHA detected in this fraction is caused by leakage from mitochondria. Based on the relative strength of the signal, we estimate that 35% of Cox23p-ΔHA is in mitochondria and 65% is in the cytosol (Fig. 5B).

The mitochondrial compartment in which Cox23p-ΔHA is located was determined by hypotonic lysis of the mitochondria and treatment of the mitochondria and resulting mitoplasts with proteinase K. Like cytochrome b2, Cox23p-ΔHA is a soluble component of the intermembrane space and is released from mitochondria when they are converted to mitoplasts (Fig. 5C). The resistance to proteinase K of the soluble matrix marker α-ketoglutarate dehydrogenase in the mitoplasts confirmed the intactness of the mitoplasts and excluded the absence of Cox23p-ΔHA in this fraction being caused by leakage from the matrix.

**Mitochondrial COX23 and Cox17p Are Not Complexed to Each Other**—The requirement of both Cox23p and Cox17p for cytochrome oxidase assembly indicated that the two proteins do not have overlapping functions. The dual requirement for cytochrome oxidase assembly also suggested that they are either components of the same complex or that they function sequentially in a common pathway involved in maturation of the CuA center.
in the high imidazole wash (Fig. 6A). Cox23p-HA, even though present at a lower concentration than Cox17p-HA-His in this strain, was recovered in the flow-through and the low imidazole wash. When a lysate obtained from BWΔCOX17/23/ST4/52 expressing both Cox17p and Cox23p with the HA tag only was similarly treated, neither protein was adsorbed to the beads (Fig. 6B), confirming that the polyhistidine tag on Cox17p is essential for its binding to the Ni-NTA column.

DISCUSSION

Cox23p is a soluble protein of the mitochondrial intermembrane space but is also present in the cytosol. It is homologous to Cox17p, which was previously shown to have the same subcellular distribution (3). The sequence similarity to Cox17p is confined to the carboxyl half of Cox23p, which appears to be the functionally important region because a truncated gene expressing the sequence starting from residue 76 does not impair its ability to complement the cox23 mutant.

Cox23p is required for mitochondrial copper homeostasis. This is manifest in the rescue by exogenous copper of the cox23 mutant transformed with COX17 on a high copy plasmid. Cox17p was initially proposed to act as a shuttle for the delivery of copper from the cytoplasm to the mitochondrial intermembrane space (3). Recent evidence showing that Cox17p is tethered to the inner membrane through a transmembrane domain is able to complement a cox17 mutant has cast doubt on the idea that this protein acts as a shuttle (29). Independent of the manner in which it works, based on extensive mutational analysis and its location inside of mitochondria, Cox17p has been linked to maturation of the Cu₃ center (3, 4). Cox23p, therefore, is likely to be involved in this important aspect of cytochrome oxidase assembly as well.

Even though Cox23p and Cox17p do not have overlapping functions, their homology and the conservation in Cox23p of the cysteine residues known to be the copper ligands of Cox17p (27) suggest that the two proteins function in an analogous manner. In the absence of evidence for a physical association between Cox23p and Cox17p, the most likely explanation of why both proteins are required for the expression of cytochrome oxidase is that they are components of a common pathway. This is consistent with evidence showing a genetic interaction between the two. The fact that COX17 is a high copy suppressor of the cox23 mutant but not the converse further argues that Cox17p functions downstream of Cox23p. These observations raise the possibility that Cox23p and Cox17p act sequentially in a mitochondrial copper transfer/distribution pathway with cytochrome oxidase as one or perhaps the sole target.

We have recently reported another protein, designated Cox19p, with properties similar to Cox23p and Cox17p (including its localization and requirement for cytochrome oxidase assembly) (30). Cox19p is homologous to Cox17p but has no sequence similarity to Cox23p. It is conceivable that Cox19p is another member of this pathway.

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**COX23, a Homologue of COX17, Is Required for Cytochrome Oxidase Assembly**
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