Isolation and Characterization of COX12, the Nuclear Gene for a Previously Unrecognized Subunit of Saccharomyces cerevisiae Cytochrome c Oxidase*

Arthur E. P. LaMarche†, Marina I. Abate‡, Samuel H. P. Chan§, and Bernard L. Trumpower††

From the †Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and the ‡Department of Biology, Syracuse University, Syracuse, New York 13244

We have cloned and sequenced COX12, the nuclear gene for subunit VIb of Saccharomyces cerevisiae cytochrome c oxidase. This subunit, which was previously not found in cytochrome c oxidase purified from S. cerevisiae, has a deduced amino acid sequence which is 41% identical to the sequences of subunits VIb of bovine and human cytochrome c oxidases.

The chromosomal copy of COX12 was replaced with a plasmid-derived copy of COX12, in which the coding region for the suspected cytochrome oxidase subunit was replaced with the yeast URA3 gene. The resulting Ura+ deletion strain grew poorly at room temperature and was unable to grow at 37 °C on ethanol/glycerol medium, whereas growth was normal at both temperatures on dextrose. This temperature-dependent, petite phenotype of the deletion strain was complemented to wild-type growth with a single copy plasmid carrying COX12.

Cytochrome c oxidase activity in mitochondrial membranes from the cox12 deletion strain is decreased to 5–15% of that in membranes from the wild-type parent, and this activity is restored to normal when the cox12 deletion strain is complemented by the plasmid-borne COX12. Optical spectra of mitochondrial membranes from the cox12 deletion strain revealed that optically detectable cytochrome c oxidase is assembled at room temperature and at 37 °C, although the heme a + a' absorption is diminished approximately 50%.

The N-terminal amino acid sequence of the protein encoded by COX12 is identical to the N-terminal sequence of a subunit found in yeast cytochrome c oxidase purified by a new procedure (Taanman, J.-W., and Capaldi, R. A. (1992) J. Biol. Chem. 267, 22481–22485). We conclude that COX12 encodes a subunit of yeast cytochrome c oxidase which is essential during assembly for full cytochrome c oxidase activity but apparently can be removed after the oxidase is assembled, with retention of oxidase activity. This is the first instance in which deletion of a subunit of cytochrome c oxidase results in assembly of optically detectable

cytochrome c oxidase but having markedly diminished activity.

Cytochrome c oxidase is the terminal electron acceptor of the respiratory chain in eukaryotes and some bacteria. The bacterial enzyme is composed of 3 subunits (1, 2), but the eukaryotic enzyme complex is composed of 7–13 subunits (3), depending upon the species. The three largest subunits of the eukaryotic complex are encoded by the mitochondrial genome and are homologous to the bacterial subunits (1). Although there is some question as to which of the three subunits contain the copper, there is general agreement that the redox active heme and copper prosthetic groups are held within the three largest subunits (4). Because of this, these three subunits are thought to be the catalytic core of the enzyme.

The remaining 4–10 subunits of the eukaryotic cytochrome c oxidase complexes, all of which are nuclear encoded, are not known to contain any redox prosthetic groups. Measurements of electron transfer and proton translocation activities with the purified bacterial and eukaryotic enzymes have failed to reveal any functional differences which might be ascribed to the extra subunits in the latter (3). The function of these subunits is thus unknown.

Until now, the yeast cytochrome c oxidase complex was thought to contain only six nuclear encoded subunits, all of which have been cloned and sequenced (6). We report here the cloning and sequencing of a yeast nuclear gene which encodes a protein that is highly homologous to subunit VIb of bovine and human cytochrome c oxidase complexes. A corresponding subunit VIb was previously not found in the purified yeast enzyme. This subunit apparently can be removed from the yeast complex with little or no effect on the ability of the enzyme to oxidize cytochrome c, since an active yeast enzyme can be purified without this subunit. To determine the role played by subunit VIb we have created and characterized a null strain for this subunit. The deletion strain assembles optically detectable cytochrome c oxidase, but the turnover number is severely reduced.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, chromosomes isolated from Saccharomyces cerevisiae strain YPH80, and Klenow fragment were purchased from New England BioLabs. Polynucleotide kinase and the Sequenase Kit were purchased from U.S. Biochemical Corp. PhBlasctect II sk-, Nuc-trap, and Prime-it were purchased from Stratagene. NENSORB was purchased from Du Pont. Erase-a-Base was purchased from Promega. [α-32P]dCTP, α,α'-dATP, and γ,γ'-ATP were purchased from Amersham Corp. SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Urea and agarose (Ultra Pure) were purchased from Bethesda Re-

* This work was supported by a grant-in-aid from the American Heart Association and the New York State Affiliate of the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M98332.

† To whom correspondence should be addressed. Dept. of Biochemistry, W. Vail Bldg., Dartmouth Medical School, Hanover, NH 03755. Tel.: 603-650-1621; Fax: 603-650-1389.

‡ To whom correspondence should be addressed. Dept. of Biochemistry, W. Vail Bldg., Dartmouth Medical School, Hanover, NH 03755. Tel.: 603-650-1621; Fax: 603-650-1389.
search Laboratories. Nytran membranes with a 0.2-μm pore size were obtained from Schleicher & Schuell. Glass beads (0.5 mm) were obtained from Biospec Products. Horse heart cytochrome c (type III) was obtained from Sigma.

**Cyclophilin A**—*S. cerevisiae* strain W303-1A (MATa, ade2-1, his3-11, leu2-3, 112, trpl-1, urs3-1, can1-100) was a gift from Dr. Alexander Tzagoloff (Columbia University). Yeasts were grown either on YPD (1% yeast extract, 2% peptone, 2% dextrose), YPEG (1% yeast extract, 2% peptone, 4% ethanol, 3% glycerol), synthetic dextrose, or on complete minimal dropout media lacking uracil. 0.175 mM “dropout powder” lacking uracil, 0.5% (NH₄)₂SO₄, and 2% dextrose (7).

**Purification of Subunit VIb**—The protein here identified as subunit VIb of yeast cytochrome c oxidase was obtained as a side product during the isolation of yeast mitochondrial ATPase inhibitor (5) 80°C. The isolation procedure was modified from the one used to isolate ATPase inhibitor from beef heart (8).

Strain S288C yeast were grown in 10 liters of YPD medium for 36–48 h at 30°C with constant aeration and harvested by centrifugation. A 300-ml suspension of yeast cells in 0.25 M mannitol, 20 mM Tris acetate, 1 mM EDTA, pH 7.5, was mixed with 150 ml of 0.5-mm glass beads and homogenized in a glass bead beater for a total of 10 min in 2-min intervals while maintaining the suspension at 4°C. After removing the settled glass beads, the suspension was centrifuged at 3,000 × g for 5 min to collect unbroken cells, which were resuspended and homogenized with glass beads as above. The supernatants were combined, debris and unbroken cells were removed by centrifugation at 15,000 × g for 20 min. The mitochondrial pellet was washed with mannitol-Tris buffer and then suspended in 0.25 M sucrose.

Mitochondria were diluted to 10 mg/ml in 0.15 M sucrose, 2 mM EDTA, 5 mM Tris sulfate and titrated to pH 11.6-11.7 within 75 s by adding 1 M KOH and then were incubated for 2.5 min with constant stirring at 4°C. The mitochondria suspension was then adjusted to pH 5.4 with 1 M acetic acid and incubated for 1 min at 4°C, after which it was neutralized to pH 7.4 with 1 M KOH.

After centrifugation at 12,000 × g for 10 min, solid ammonium sulfate (2.43 g/10 ml) was added to the supernatant, which was then centrifuged for 10 min at 12,000 × g. The pellet was discarded, and additional ammonium sulfate (2.0 g/10 ml) was added to the supernatant. After centrifugation as above, the pellet was dissolved in 0.25 M sucrose. Ice-cold 50% trichloroacetic acid was added to a final concentration of 10%, and the mixture was immediately centrifuged at 20,000 × g for 5 min. The pellet was dissolved in water, the pH was adjusted to 5.0 with 1 N KOH, and the mixture was centrifuged at 15,000 × g for 5 min. The pellet was discarded, and ammonium sulfate (2.0 g/10 ml) was added to the supernatant. This mixture was centrifuged at 12,000 × g for 10 min, and the resulting pellet was dissolved in 0.25 M sucrose. The dissolved pellet was incubated at 95°C for 3 min, chilled in an ice bath, and centrifuged at 12,000 × g for 10 min. The clear supernatant was dialyzed against distilled water, and the resulting dialysate was lyophilized (lyophil) to a small volume.

The partially purified ATPase inhibitor fraction was dissolved at approximately 5 mg/ml in 10 mM sodium acetate, pH 6.0, and injected into a 4.6-mm × 7.5-cm Ultrapore RPSC cation exchange high pressure liquid chromatography column (Beckman), which was equilibrated in 10 mM sodium acetate, pH 6.0. The column was eluted with a linear gradient from 0 to 1 M NaCl in 10 mM sodium acetate, pH 6.0, and fractions monitored by 280 nm absorbance. Four protein peaks eluted from the column and were analyzed by SDS-polyacrylamide gel electrophoresis (9). In addition to three polypeptides corresponding to the inhibitor and inhibitory-associated polypeptides (10, 11), an additional protein which migrated slightly faster than horse heart cytochrome c on SDS-polyacrylamide gel electrophoresis was also observed. The protein was transferred electrophoretically to a polyvinylidene difluoride membrane (12). N-terminal amino acid sequencing of the unknown protein was performed by Drs. L. Lane and David Andrews of the Harvard Biological Laboratories (Cambridge, MA) using an Applied Biosystems 470A gas phase sequencer.

**Oligonucleotide Construction and Purification**—A 25-base oligonucleotide probe was designed on the basis of the sequence of the first 9 amino acids of the N terminus of purified subunit VIb, as shown in Fig. 1a. The sequence of the oligonucleotide was constructed with a yeast codon bias, which was further biased by taking into account observed codon usages for yeast nuclear encoded mitochondrial pro-

---

*Cytochrome c oxidase subunit VIb gene*

*Cloning of COX12*—The COX12-containing clone was isolated from a yeast strain S288C genomic library in a YEp24-based plasmid constructed by Dr. Mark Rose (Princeton University). Clone C1, shown in Fig. 1b, was isolated by colony lift hybridization (13) of approximately 40,000 Escherichia coli colonies. After washing the nitrocellulose filters twice at 42°C in 2× SSC (0.15 M NaCl, 0.015% sodium citrate, pH 7.0) and 0.025% BLOTTO (5% nonfat dried milk, 0.02% sodium azide) for 30 min, a single colony was detected by the oligonucleotide probe.

*Subcloning, DNA Manipulations, and Sequencing of COX12*—Clone C1 was digested with several restriction enzymes, and the binding of the oligomer was localized to a 1.7-kbp 1 XhoI fragment. For sequencing, a subclone, pRS315-9kd, was constructed by isolating a 3.4-kbp Xbal/BamHI fragment of clone C1 (Fig. 1b) containing COX12 and inserting this fragment into pRS315 cut with Xhol and BamHI (14). pRS315-9kd was digested with ScaI and XhoI or Aat and BamHI to obtain nested deletions in both directions over the length of the insert using the Erase-a-Base system as recommended by the manufacturer. Southern hybridization (15) was performed to identify the smallest clone which still bound the oligomer. Sequencing was performed using the Sequenase kit as directed by the manufacturer using the RACE method (16). This fragment was expected to integrate at the COX12 locus by the TENS method (16).

*Yeasts*—Yeasts were transformed after treatment with lithium acetate (17). *E. coli* strain DH5α was used for amplification of plasmids and was made competent for transformation with dimethyl sulfoxide as per Hanahan protocol 2 (18). Plasmids were isolated according to Maniatis and co-workers (13) or by the TENS method (16).

*Computer Software Analysis—MacVector software, run on a Macintosh computer, was used to identify restriction endonuclease sites, open reading frames, and amino acid composition of the gene.*

*GenBank and SwissPro data banks were searched using the Genetics Computer Group Sequence Analysis software package located on a VAX117-80 computer at Dartmouth College Computing Center.*

*Amino acid sequence homologies were analyzed with the FASTA program (19).*

*Construction of a coxl2 Deletion Strain—A coxl2 deletion strain was constructed by removing most of the promoter and 95% of the coding region between the ECRV sites which are internal to the EcoRI/Xholl fragment (see Fig. 1b). For construction of a deletion plasmid, pRS315-9kd was digested with EcoRI and Xhol, and the EcoRI/Xhol fragment containing the COX12 gene was subcloned into a λgt10 vector (20) and purified. The vector was digested with EcoRV to remove the COX12 gene. The yeast URA3 gene was removed from pUC18-URA3/K211 (a gift from Dr. Alexander Tzagoloff, Columbia University) on a HindIII cassette and the ends made blunt by filling in with Klenow. The blunted 1.1-kbp HindIII fragment of URA3 was subcloned into the EcoRI site of pRS315 and then digested with EcoRI and Xhol to liberate the linear yeast DNA and used to transform W303-1A (17). This fragment was expected to integrate at the COX12 locus by homologous recombination. Selection for uracil prototrophy identified transformants in which the wild-type COX12 was presumably replaced with the URA3-deleted copy. The presumed deletion strain was named APL-1A. Complementation of the deletion strain was checked using pRS315-9kd and clones obtained in the nested deletion protocol. Plasmids were transformed (17) into APL-1A by selecting for leucine prototrophy, and transformants were checked for their ability to grow on nonleucine-containing carbon sources.*

*Yeast DNA was prepared according to the method of Hoffman and Winston (20). Genomic DNA analysis was done by the method of Southern (15).* DNA was digested with *XhoI*, separated on a 1% agarose gel, and transferred to a nylon membrane. The blot was blocked for 3 h in 6 × SSC and 0.05% BLOTTO and then probed in the same solution for 12 h using the 1.7-kbp *XhoI* fragment of pRS315-9kd labeled using a Prime-it kit. The blot was washed twice in 2 × SSC and 0.1% SDS for 15 min at 65°C and then twice in 0.1 × SSC, 0.1% SDS for 15 min at 65°C. Autoradiography was performed at −80°C with Kodak XAR-5 film and a Du Pont Cronex Lightning Plus intensifying screen.

**Cytochrome c oxidase subunit VIb gene**

**Chromoblot Analysis—Chromosomes from *S. cerevisiae* strain**

The abbreviations used are: kbp, kilobase pair(s); bp, base pair(s).
YEP80, purchased from New England Biolabs, were separated on a 1% agarose gel and transferred to a nylon membrane. The membrane was probed with a 1.7-kbp *XhoI* fragment of pRS315-9kd using the conditions used for the Southern hybridization of the genomic DNA.

**Mitochondrial Preparations**—Yeast cultures were grown overnight in synthetic dextrose medium supplemented with appropriate nutrients, and 500-μl aliquots of the suspended cells were then spread on YPD plates and grown for 2 days at 24 or 37°C. Twenty plates of cells were harvested for each strain from each temperature and washed twice with distilled water. All subsequent steps were performed at 4°C. The pellet of yeast cells from 20 plates was resuspended in 2 ml of 400 mM mannitol, 50 mM Tris, 2 mM EDTA, pH 7.4 for isolation of mitochondria (21). An equal volume of 0.5-mm glass beads was added to the resuspended cells. The cells were broken by mixing with the beads on a vortex mixer for 1 min and returned to an ice bath for 1 min. This step was repeated 10 times, after which 10 ml of the mannitol-Tris buffer was added, the slurry was vortexed for 1 min and centrifuged at 3,000 × g for 10 min. The supernatant was removed and saved. The pellet was resuspended in another 15 ml of mannitol-Tris buffer and centrifuged at 3,000 × g for 10 min.

The supernatants were combined and centrifuged at 12,000 × g for 20 min. The 12,000 × g supernatant was discarded, and the pellet was resuspended in 25 ml of mannitol-Tris buffer and centrifuged at 12,000 × g for 20 min. The supernatant was discarded, and the pellet was resuspended in 25 ml of 150 mM KCl, 25 mM K₂HPO₄, pH 7.0, homogenized, and centrifuged at 12,000 × g for 20 min. The pellet was resuspended, homogenized, centrifuged again at 12,000 × g for 20 min, and then suspended in 5 ml of the phosphate buffer. Protein concentrations were determined by the method of Lowry et al. (22), as modified by Markwell et al. (23).

**Growth and Activity Measurements**—Yeast were grown in either YPD or YPEG medium. Growth rates were measured by monitoring the turbidity of 250-ml Liquid cultures, using a Klett spectrophotometer with a green filter. Ubiquinol-cytochrome c oxidoreductase activities were measured at 23°C in 40 mM Na₂HPO₄, 20 mM malonic acid, 0.5 mM EDTA, pH 7.4, containing 40 μM cytochrome c. After determining the nonenzymatic rate of cytochrome c reduction following addition of 66 μM 2,3-dimethoxy-5-methyl-6-nonyl-1,4 benzoquinol to the assay mixture, the reaction was initiated by adding mitochondrial membranes, and reduction of cytochrome c was followed at 550 nm. Cytochrome c oxidase activities were measured at 23°C in 40 mM K₂HPO₄, pH 6.65, containing 20 μM reduced cytochrome c and 0.1% lauryl maltoside. Oxidation of cytochrome c was followed by measuring the decrease in absorbance at 550 nm.

**Spectroscopy**—Optical absorption spectra of mitochondrial membranes were obtained at room temperature on an Aminco DW-2A dual wavelength scanning spectrophotometer equipped with a Nicolet digital densitometer and a x-y recorder. The spectrophotometer was operated in the split beam mode with a 1 nm band pass. Membranes were suspended in 25 ml of 150 mM KCl, 25 mM K₂HPO₄, 1% Triton X-100, pH 7.0, and reduced or oxidized by the addition of a small amount of sodium dithionite or potassium ferricyanide, respectively.

**RESULTS**

**Isolation of COX12**—The protein encoded by COX12 was isolated as an unknown protein in a yeast ATPase inhibitor preparation. The N-terminal sequence of the first 14 amino acids of the unknown protein is shown in Fig. 1a. A single 25-base oligomer, also shown in Fig. 1a, was constructed on the basis of the first 9 amino acids of the unknown protein. This oligomer was used to screen approximately 45,000 *E. coli* colonies from a yeast genomic library in YEp24-C1 clone, YEp24-C1, consistently bound the labeled probe. Plasmid DNA from YEp24-C1 was digested with several restriction enzymes, separated on an agarose gel, and transferred to a nylon membrane. Binding of the oligomer was thus localized to the 1.7-kbp *XhoI* fragment. A partial restriction map of clone YEp24-C1 containing the COX12 gene isolated from a yeast genomic library. The N-terminal amino acid sequence of subunit VIb is shown in *panel a*. Amino acids underlined were ambiguous in the sequencing reaction. The biased 25-base oligomer designed as a probe is shown beneath the amino acid sequence. A partial restriction map of clone YEp24-C1, including COX12, is shown in *panel b*. The yeast genomic library, obtained from Dr. Mark Rose, was made from a Sau3A1 partial digest of yeast strain S288C genomic DNA inserted into the BamHI site of YEp24. The clone containing COX12 was isolated by probing the library with the 25-base oligomer. Binding of the oligomer was localized to a 1.7-kbp *XhoI* fragment, which is expanded and shown below the plasmid map.

Nested deletions were constructed in both directions across the insert. To minimize the amount of DNA to be sequenced, the clones obtained from the nested deletion procedure were also screened with the oligomer. Binding of the probe to a subset of the nested deletion plasmids was thus further localized, after which appropriate plasmids were sequenced as described above. The sequencing strategy is shown in Fig. 2.

**Characterization of the COX12 Structural Gene**—The nucleotide sequence of approximately 900 bases, arrived at by the sequencing strategy shown in Fig. 2, is shown in Fig. 3. This section of the clone bound the oligomer probe. Analysis of the nucleotide sequence revealed an open reading frame which included a sequence of amino acids that closely, but not exactly, matched that of the N-terminal sequence obtained from the unknown protein.

Gas phase sequencing had indicated that the N-terminal sequence of the unknown protein was Ala-Asp-Gln-Glu-Asn-Ser-Pro-Pro-Thr-Val-Gly-Ile-Asp. Because of the small amounts of protein submitted for sequencing, Ser-6, Pro-8, Thr-9, and Thr-10 were considered ambiguous (Fig. 1a). The open reading frame in the nucleotide sequence begins with a methionine, which is followed by the sequence Ala-Asp-Gln-Glu-Asn-Ser-Pro-Leu-His-Thr-Val-Gly-Phe-Asp. Of the 14 amino acids determined from the protein sequence, only 3 do not agree with the sequence deduced from the DNA, and two of these were ambiguous in the protein sequence. The open reading frame encodes a protein of 83 amino acids having a predicted molecular mass of 9,787 daltons prior to any post-translational modification. Before the initial methionine of the open reading frame there is a stop codon. Therefore, it would appear that the mature protein has only the methionine of the N terminus removed.

The amino acid sequence of the unknown protein translated...
**Fig. 2.** Strategy used in sequencing COX12. The location and direction of COX12 are marked by the solid arrow in the restriction map at the top. Restriction sites used in subcloning are listed above the line. Clones that were sequenced and tested for complementation of the APL-IA deletion strain are shown by the bracketed solid lines, in which the length of sequence obtained from each clone is indicated by an arrowhead. The names of the clones are indicated to the left. Clones that complemented the deletion strain are indicated by +, those that were unable to complement by a -.

Clone B-376, scored as +/−, partially complemented the deletion strain to growth on ethanol/glycerol, in that the growth rate was markedly slower than the wild-type parent. Clone B-376 contains the entire structural gene for COX12, but the majority of the promoter has been deleted.

**Fig. 3.** Nucleotide sequence of COX12, its flanking regions, and amino acid sequence of the COX12-encoded protein as deduced from the gene. The nucleotide sequence is numbered to the left, with the A of the initial methionine codon numbered as 1. The amino acid sequence of the COX12-encoded protein is also numbered to the left, in parentheses, with the initial methionine of the open reading frame numbered as 1. Restriction endonuclease sites used for subcloning are indicated beneath the nucleotide sequence and underlined. The putative HAP2/HAP3 binding sites are also underlined.

The protein encoded by COX12 is homologous to the subunits VIb from bovine and human cytochrome c oxidases. Shown are the amino acid sequence of the yeast protein encoded by COX12 and the amino acid sequences of the subunits VIb from bovine and human cytochrome c oxidases. Regions of identity among the three sequences are enclosed by solid lines, and regions in which the sequences are similar if conservative replacements are allowed are enclosed by dotted lines. The yeast protein encoded by COX12 is 41% identical to the subunits VIb of the bovine and human cytochrome c oxidases and is 77% similar if conservative substitutions of amino acids are allowed for. The arrows point to the 4 cysteines which are conserved in the three proteins.

In addition to the overall sequence similarity, the yeast and mammalian subunits VIb have 4 cysteine residues which are conserved within their sequences (Fig. 4). The yeast protein differs from the mammalian subunits in that the latter have acetylated N termini, and the former does not. Examination of the predicted secondary structures (25) of the yeast and mammalian subunits VIb indicates that the three proteins have in common two very hydrophilic domains with a high probability of being located at the surface of the protein: one near the N terminus, extending from Arg-17 through Trp-28 of the yeast protein, and a second near the C terminus, extending from Glu-65 through Lys-73. Although there is a relatively high degree of conservation of amino acid sequence between the three proteins, there are marked differences in the nature of the charged amino acids. Consequently, the calculated isoelectric points of the yeast, bovine, and human subunits VIb are 4.96, 9.31, and 7.00, respectively. This simple comparison suggests that the net charge on the two apparently
conserved hydrophilic domains is inconsequential to the function of those domains.

There are several predicted $\alpha$-helixes in the three proteins but only a single helix of significant length which appears to be common to the three proteins. This helix is approximately in the middle of the three proteins, extending from Cys-37 through Val-50 of the yeast protein. There does not appear to be any region of sequence within subunit VIb of sufficient length and hydrophobicity to comprise a membrane spanning domain. Taken together, the secondary structure predictions suggest that subunit VIb is located on the aqueous surface of cytochrome $c$ oxidase and held there by protein-protein interactions, rather than through a membranous tether. This interpretation is consistent with the apparent ease with which this subunit is dissociated from the membranous complex during purification.

Construction of a coxl2 Deletion Strain—Since subunit VIb apparently can be removed from cytochrome $c$ oxidase during purification with little, if any, detriment to the activities of the purified enzyme, we wanted to determine the properties of cytochrome $c$ oxidase in yeast lacking the ability to synthesize this subunit. For this purpose we constructed a yeast strain in which the chromosomal copy of COX12 was deleted and replaced with the URA3 gene.

Six transformants were obtained by selection for uracil prototrophy. Each isolate was grown overnight in medium lacking uracil, after which growth rates of the transformants were checked on YPD and YPEG. Four of the six transformed strains, APL-1A, 1B, 1C, and 1D, had a markedly decreased rate of growth on YPEG at 24, 30, and 37 °C but grew normally at these temperatures on YPD. This growth impairment on nonfermentable carbon sources became more severe at the higher growth temperatures, as shown below.

Analysis of the COX12 Genomic Locus in the coxl2 Deletion Strains—To ascertain that the growth impairment on nonfermentable carbon sources was linked to the anticipated deletion of the COX12 gene, chromosomal DNA was prepared from each of the six transformants, digested with selected restricted enzymes, and compared with the parental DNA by Southern hybridization with a fragment of the cloned COX12 gene.

When DNA from the parental strain is cut with XhoI and probed with the COX12 gene, a single DNA fragment with a mobility corresponding to approximately 1.7 kbp is detected by the probe, as shown in Fig. 5. Although the exact size expected for this fragment has not been determined, since the sequencing was not extended up to the XhoI site distal to the 3' end of COX12, this apparent size agrees with the size of this fragment expected from the restriction map of the COX12 clone (see Fig. 1b). When DNA from the four strains which had decreased growth rates on ethanol/glycerol were similarly digested and probed, a single band of 2.3 kbp was detected (Fig. 5). This is in good agreement with the size expected of this fragment if the 400-bp EcoRV fragment at its 3' end of COX12 (see Figs. 1b and 3) is replaced by the 1.1-kbp URA3 gene at the chromosomal locus of COX12.

When the 400-bp EcoRV fragment of COX12 was used as a probe, it detected a single 400-bp EcoRV fragment in the parental DNA, whereas no EcoRV fragment was detected in DNA from the four strains which grew poorly on ethanol/glycerol (results not shown). This pattern is also as expected if the 400-bp EcoRV segment of the COX12 gene at its chromosomal locus is replaced with the URA3 gene. From these results we conclude that the COX12 structural gene was replaced by the URA3 gene in the four strains which grew poorly on ethanol/glycerol and that the poor growth on nonfermentable carbon sources resulted from this replacement. The Southern hybridization also establishes that COX12 is present in the W303-1A genome as a single copy gene.

The linkage between deletion of COX12 and the poor growth on nonfermentable carbon sources was also confirmed genetically by back-crossing APL-1A, one of the yeast strains which grew poorly on ethanol/glycerol, to a wild type, ura3-
pulsed-field gel electrophoresis and transferred to nylon. The blot when these were digested with XhoI and probed with the

on the nonfermentable carbon source (results not shown).

with pRS315-9kd, a CEN plasmid carrying the COX12 gene.

tion of COX12, the deletion strain APL-1A was transformed

strain. When this diploid was sporulated, the spores exhibited

2:2 linkage in which the ura+ spores failed to grow at 37 °C

Carrying the COX12 Gene—To confirm further that the
growth phenotype of the deletion strain was caused by dele-

Complementation analysis we conclude that COX12 consists

Chromosomal DNA from the two ura+ transformants which
grew normally on ethanol/glycerol exhibited a single band at

Localisation of COX12 to Chromosome XII—Yeast chromo-
somes from S. cerevisiae strain YPH80 were separated by

Ubiquinol-Cytochrome c Oxidoreductase and Cytochrome c
Oxidase Activities of the Deletion Strain—To establish the
biochemical basis for the inability of the cox12 deletion strain
to grow on nonfermentable carbon sources, mitochondrial
membranes were isolated from the parental strain and the
cox12 deletion strain, and their ubiquinol-cytochrome c oxi-
doreductase and cytochrome c oxidase activities were com-
pared. As shown in Fig. 8a, the ubiquinol-cytochrome c oxi-
doreductase activity of mitochondrial membranes from the
deficiency strain APL-1A was essentially identical to that of
membranes from the parent strain W303-1A. This comparison
indicates that the cytochrome bc complex is unaffected by
the deletion of COX12.

Deletion of COX12 results in a marked decrease in cyto-
chrome c oxidase activity, as shown in Fig. 8b. When expressed
on a per mg of protein basis, the oxidase activity in APL-1A
is approximately 7% of the oxidase activity of the parental
strain when grown at 24 °C. When the deletion strain was
complemented with the single copy pRS315-9kd plasmid,
Ubiquinol-cytochrome c reductase and cytochrome c oxidase activities of mitochondrial membranes from wild-type yeast and a cox12 deletion strain. The tracings in panel a show the reduction of cytochrome c versus time by mitochondrial membranes isolated from the W303-1A parental strain and the cox12 deletion strain APL-1A. At the times indicated ubiquinol substrate (S) was added to initiate the nonenzymatic rate, after which the reaction was initiated by the addition of mitochondrial membranes (M). The tracings in panel b show oxidation of cytochrome c by mitochondrial membranes isolated from W303-1A and the cox12 deletion strain APL-1A. Oxidation of reduced cytochrome c was initiated by the addition of mitochondrial membranes (M). The yeasts were grown on YPD plates at room temperature and mitochondrial membranes isolated as described under "Experimental Procedures.”

Optical spectra of mitochondrial membranes from wild-type yeast and a cox12 deletion strain. The optical spectra were obtained on the same preparations of mitochondrial membranes as used for the activity measurements in Fig. 8.
Cytochrome c oxidase subunit VIb gene
carrying COX12, the plasmid restored the oxidative activity to that of the parental strain, coincident with restoration of the ability of the strain to grow on nonfermentable carbon sources (results not shown).

Optical Spectra of the Cytochromes of the Respiratory Chain of the Deletion Strain—Optical spectra of the cytochromes of mitochondrial membranes of the parental and deletion strains, suspended at identical protein concentrations, are shown in Fig. 9. In comparison to membranes from the parental strain, there appears to be a slight increase in concentrations of cytochromes b and c1 in APL-1A. Similarly, prior to washing the membranes with salt, we observed an apparent increase in the amount of cytochrome c associated with the mitochondrial membranes isolated from APL-1A (results not shown). These preliminary observations suggest that the loss of cytochrome c oxidase activity resulting from deletion of COX12 may lead to a compensatory increase in the amount of cytochrome bc1 complex and cytochrome c. Further experimentation is in progress to explore this possibility.

Most notably, the optical spectra reveal that cytochromes a and a2 are present in mitochondrial membranes from APL-1A (Fig. 9). These spectra were obtained on the same preparations of mitochondrial membranes which were used for the activity measurements in Fig. 8 and establish that optically detectable cytochrome c oxidase is assembled in APL-1A but that the enzyme has only marginally detectable activity when subunit VIb is genetically deleted. Closer examination of the spectra in Fig. 9 reveals that the amount of optically detectable cytochromes a + a2 in APL-1A is reduced to about one-half the level of that in membranes of the parental strain. Taken together the activity measurements (Fig. 8) and optical spectra (Fig. 9) indicate that the heme groups are assembled into the oxidase complex in the absence of subunit VIb, but that the turnover number of the enzyme is reduced from 170 s⁻¹ in the parental strain to 26 s⁻¹ in APL-1A. Similarly, at 37 °C the deletion strain assembled a spectrally detectable but enzymatically inactive cytochrome c oxidase (results not shown).

DISCUSSION
We have cloned and partially characterized COX12, the single copy nuclear gene for subunit VIb of yeast cytochrome c oxidase. COX12 encodes a 9,787-dalton protein from which only the N-terminal methionine is post-translationally removed during import and assembly of this subunit into cytochrome c oxidase complex. Subunit VIb of cytochrome c oxidase thus resembles several of the small subunits of the cytochrome bc1 complex (34) and some of the smaller subunits of cytochrome c oxidase (6) in lacking a cleavable, N-terminal presequence. Presumably the mitochondrial targeting sequences of these small proteins are internal in the mature proteins.

Subunit VIb is essential for the assembly of a fully active cytochrome c oxidase complex. However, the subunit is apparently readily lost from cytochrome c oxidase during purification of the enzyme with detergent, and the subunit VIb-depleted enzyme remains active. What function, if any, subunit VIb fulfills in the activity of cytochrome c oxidase and whether there may be previously unrecognized differences in the activities of cytochrome c oxidase lacking and containing subunit VIb remain to be established.

Deletion of COX12 and the attendant absence of subunit VIb results in a temperature-sensitive petite phenotype linked to a marked loss of cytochrome c oxidase activity. The most notable feature of the cytochrome c oxidase defect is that optically detectable oxidase is assembled. To our knowledge this is the first instance in which deletion of the nuclear gene for a subunit of cytochrome c oxidase permits assembly of an oxidase complex having very little activity. It remains to be established whether all of the other oxidase subunits are present in the subunit VIb-deficient enzyme, and whether the copper redox centers are normally assembled in the inactive enzyme. Such experiments are now in progress.

REFERENCES
33. Trueblood, C. E. Y. Yeast 6, 271-297
Isolation and characterization of COX12, the nuclear gene for a previously unrecognized subunit of Saccharomyces cerevisiae cytochrome c oxidase.

A E LaMarche, M I Abate, S H Chan and B L Trumpower


Access the most updated version of this article at http://www.jbc.org/content/267/31/22473

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/267/31/22473.full.html#ref-list-1