Cloning and Characterization of PET100, a Gene Required for the Assembly of Yeast Cytochrome c Oxidase*

Cynthia Church, Christine Chapon, and Robert O. Poyton‡

From the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

(Received for publication, January 29, 1996, and in revised form, May 8, 1996)

The biogenesis of cytochrome c oxidase in Saccharomyces cerevisiae requires a protein encoded by the nuclear gene, PET100. Cells carrying a recessive mutation (pet100-1) in PET100 are respiratory deficient and have reduced levels of cytochrome c oxidase activity. The PET100 gene has been cloned by complementation of pet100-1, sequenced and disrupted. PET100 is located adjacent to the PDC2 gene on chromosome IV and contains an open reading frame of 333 base pairs. The PET100 protein contains a possible membrane-spanning segment and a putative mitochondrial import sequence at its NH₂ terminus. A strain carrying a null mutation in PET100 lacks cytochrome c oxidase activity and assembled cytochromes a and a₃, but the other respiratory chain carriers are present. The respiratory-deficient phenotype of this strain is not rescued by added hemin or heme A. These findings indicate that the mutation is specific for cytochrome c oxidase and does not affect the biosynthesis of heme A. In addition, mitochondria from the strain carrying a null mutation in PET100 contain each of the subunit polypeptides of cytochrome c oxidase. Together, these findings suggest that PET100p is not required for the synthesis or localization of cytochrome c oxidase subunits to mitochondria, but is required at a later step in their assembly into an active holoenzyme.

Cytochrome c oxidase is the terminal enzyme of the mitochondrial and some bacterial respiratory chains. This membrane protein catalyzes the final step of cellular respiration by relaying electrons from reduced cytochrome c to molecular oxygen, forming water. The energy derived from this electron transfer drives the formation of a proton gradient. Prokaryotic cytochrome c oxidases consist of two subunits which carry four redox-active centers (heme a, heme a₃, Cuₐ, Cuₐ₃) and one or two additional subunits. In eukaryotes, structural and functional homologues of three of the bacterial subunits are products of the mitochondrial genome. Two of these, subunits I and II, form the functional center of the enzyme. In addition to the mitochondrially encoded subunits, eukaryotic cytochrome c oxidases contain between four and ten subunits. The literature suggests that three additional polypeptides are present in yeast cytochrome c oxidase preparations. At least two of these, encoded by COX12 and COX13, have partial sequence homology to polypeptides found in mammalian cytochrome c oxidases. Insofar as the nine subunit enzyme is fully active, the function of these polypeptides is unknown. As reviewed recently, it is not yet clear if these polypeptides are bona fide subunits of the holoenzyme, polypeptides required for assembly, or contaminating polypeptides that copurify with it.

Recent studies on respiration-deficient yeast mutants have shown that the biogenesis of cytochrome c oxidase depends not only on the COX structural genes but also on a large number of other nuclear genes. Among these are genes required for expression of the mitochondrial COX genes, heme A synthesis, and assembly of a functional holoenzyme. Here, we report a new nuclear gene, PET100, whose encoded protein appears to specifically affect the assembly of cytochrome c oxidase. The phenotype of a PET100 null mutant indicates that PET100p, like several other recently reported proteins, is not required for synthesis of subunit polypeptides or their localization to the mitochondrion but is essential for a later step in the assembly pathway.

MATERIALS AND METHODS

Strains and Plasmids—Escherichia coli strain TG1 (24) was used for the propagation of plasmid DNA. The shuttle vector P366 (25), containing a yeast genomic library, was used for doing the PET100 gene. This plasmid is derived from YEp50 (26) and contains a LEU2 gene in place of a URA3 gene as the selectable marker in yeast. The yeast genomic library in this plasmid consists of nuclear DNA, digested with Sau3AI
to yield partial fragments averaging 10–12 kb in size. These fragments were ligated into the BamHI site of the vector. For subcloning, the centromeric vectors pFL36 or pFL38 were used (27). Plasmid SK-PET100 was used to identify the ORF corresponding to PET100; it was constructed by inserting the 1.48-kb BamHI-PstI fragment from PET100 into the BamHI-PstI site in plasmid SK (28).

The yeast strains used in this study are listed in Table I. Strains J M43, J M22, and BC123 have been described previously (13, 29).

Strains B9-10B and B9-10D are derivatives of BC123 that carry a hem2 mutation (30), and A11 was found to carry a pet100-1 mutation (31) as follows. The marker was isolated, and the disruption was confirmed by Southern blot analysis. To establish that the OP1-I fragment from plasmid YEp13 (32) was inserted into the PET100 gene, was digested with BamHI and PstI. The resultingplasmid, pCC-GD100, bearing the disruption of the PET100 gene, was digested with BamHI and PstI and used to transform J M43 to Leu + prototrophy. Leu + transformants were selected and then screened for growth on glycerol media lacking leucine. The smallest complementing insert was identified by restriction mapping and subcloning (see “Results”).

DNA Preparation and Sequencing—Plasmid DNA was purified as described (36). DNA sequence analysis was performed by the dye-deoxy chain termination method (37) using a Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.). The oligonucleotide primers used were 20 bases long and corresponded to polylinker sites in plasmid pFL38. Sequencing reactions were analyzed with multiple loadings on 6% acrylamide gels (Sequagel, National Diagnostics). DNA sequences were compared with sequences in the EMBL/GenBank™ data bases.

Table I

| Strain   | Genotype                          | Source       |
|----------|-----------------------------------|--------------|
| JM43     | his4–580, trpl–289, leu2–3,112, ura3–52 | Ref. 13      |
| JM22     | his4, op1                          | Ref. 29      |
| BC123    | his4, op1, pet100–1                | Ref. 13      |
| B9–10B   | his4–580, trpl–289, leu2–3,112, pet100–1 | This study |
| B9–10D   | his4–580, ura3–52, pet100–1        | This study   |
| JM43GD100| his4–580, trpl–289, leu2–3,112, ura3–52, pet100::LEU2 | This study |
| JM8      | his4 [rpo2]                        | Ref. 29      |
| CR7      | his4–580, trpl–289, leu2–3,112, ura3–52, hem2 | This study |
| CR18     | his4–580, leu2–3, cox11             | This study   |

1 The abbreviations used are: kb, kilobase(s); kbp, kilobase pair(s); TPMD, tetramethyl-p-phenylenediamine; ORF, open reading frame; bp, base pair(s).
Cloning and Characterization of PET100

Table II

| Strain      | Growth on glycerol | Cytochrome c oxidase activity in isolated mitochondria |
|-------------|--------------------|-------------------------------------------------------|
| J M43      | +                  | 74.76 100                                             |
| B9-10D     | ≈                  | 13.90 18.60                                           |
| J M43G0100 | −                  | 0.87 0.01                                            |

Disruption of the PET100 gene results in loss of cellular cytochrome c oxidase activity

a μmol of cytochrome c oxidized per min/mg of mitochondrial protein.

Results

Phenotype of pet100-1—Strain BC123, which carries a pet100-1 mutation, was identified from a collection of cytochrome c oxidase-deficient mutants (3) isolated after mutagenesis of the parent strain, J M22, with ethyl methanesulfonate (13). BC123 is the only isolate in complementation group 30 (13). This mutant is complemented by rho0 tester strains, in particular BC123 is the only isolate in complementation group 30 (13). BC123 does not grow on nonfermentable carbon sources like glycerol, contains 4.8% of the level of cytochrome c oxidase activity present in its parental strain JM22, and has reduced levels of cytochromes aa3 (13). Both J M22 and BC123 also carry an op1 (also known as aac2 or pet9) mutation in the structural gene for the major aerobically expressed isozyme of the ADP/ATP translocator of the inner mitochondrial membrane. Because this mutation is in cis with either pleiotrophic mitochondrial deletions mutations (i.e. rho- mutations) or mutations lacking a mitochondrial genome (rho0 mutations), its presence in the parent strain favors the isolation of either nuclear (pet) or nonpleiotrophic mitochondrial (mit-) mutations (44). Rho- and rho0 mutations are lethal in an op1 genetic background. Although the presence of the op1 mutation was useful for the original isolation of nuclear encoded cytochrome c oxidase deficient mutants, its presence is inconvenient for cloning because op1 mutants, like cytochrome c oxidase-deficient mutants, are incapable of growth on nonfermentable carbon sources. Moreover, in some cases the op1 mutation contributes to the respiratory-deficient phenotype. Therefore, in order to clone PET100 and at the same time eliminate any phenotypic effects of the op1 mutation, we constructed two OP1 derivatives (B9-10B and B9-10D) of BC123. Unlike BC123, both of these strains exhibit some growth on glycerol (see below) and at least one of them, B9-10B, has higher levels of mitochondrial cytochrome c oxidase activity than BC123. This strain has 18.6% of the level of cytochrome c oxidase activity found in J M43 (Table II). This level of activity is about 4-fold higher than that reported for BC123 (13). Although the growth deficiency of B9-10B on glycerol and its cytochrome c oxidase deficiency are not as severe as those of BC123, they were sufficient for cloning the PET100 gene by transformation.

Isolation and Sequencing of PET100—PET100 was cloned by complementation of the growth deficiency of pet100-1 cells on glycerol. Specifically, pet100-1 mutant strain B9-10B was transformed with a yeast genomic DNA library carried on the LEU2-containing plasmid, p366. Out of 20,000 Leu + transformants, 37 colonies grew on nonfermentable medium. To confirm that complementation is conferred by the plasmid, and not an artifact resulting from reverstion of the pet100-1 gene to wild-type, the seven strongest colonies of the 37 candidates were picked, their DNA was amplified by transforming it into E. coli, and plasmid DNA was isolated from the bacterial transformants. Each of these seven DNAs was reintroduced into yeast pet100-1 cells, and the cells were tested for growth on glycerol. All seven candidates grew. This confirmed that the glycerol-positive phenotype was plasmid-linked and not the result of reverstion of a nuclear gene.

The average size of the nuclear DNA inserts in the complementing plasmid library was 10–12 kilobases. To localize the region of PET100 activity more precisely, BamHI restriction fragments from two of these clones (2 and 4) were subcloned into pFL vectors at the BamHI site of the polylinker region and tested for complementation. Restriction endonuclease digestion showed that fragments from the cloned DNA share a common 3-kbp Sau3A-BamHI restriction fragment (Fig. 1A). To further localize the complementing sequence, the PstI site of clone 4 was cut in the overlapping region (i.e. the 3-kbp Sau3A-BamHI fragment). This yielded two plasmids: 1) a plasmid containing a BamHI-PstI fragment of approximately 1.5 kbp (clone 9) that did not complement the pet100-1 mutation; and 2) a pet100-complementing plasmid containing a slightly smaller BamHI-PstI insert, designated as clone 10 (Fig. 1B). Clone 10 contains two oppositely oriented ORFs: 1) a complete ORF of 333 bp and 2) an incomplete ORF of 477 bp. Because the 333-bp ORF is present in its entirety, we assumed that it was the coding sequence of the complementing gene. This assumption was confirmed by inserting the BamHI-PstI fragment of clone 10 into plasmid SK, creating SK-PET100, and then adding four extra nucleotides at the unique BglII site that lies within the 333-bp ORF. This resulted in the loss of the BglII site and the addition of a stop codon in its place, inactivating the ORF. The construct containing the inactivated ORF did not complement a pet100-1 mutation, demonstrating that the 333-bp ORF corresponds to PET100. The sequence of this ORF, as well as the 5' and 3' sequences that flank it, are shown in Fig. 2. Comparisons of both the DNA sequence and the predicted protein sequence to DNA and protein sequences in the GenBank™ database revealed no significant homologies to any previously described genes or proteins. The upstream flanking region of the PET100 ORF contains one strictly conserved TATA box sequence element, between −359 and −354 bp.

The PET100 Gene Product—The protein-coding region of PET100 contains 111 codons and predicts a primary translation product with a molecular mass of 13,298 daltons. The codon bias (−0.0185) for this protein, calculated according to Benetzen and Hall (45), is characteristic of a weakly expressed gene. The PET100 protein (PET100p) has a high content of hydrophilic amino acids with more basic than acidic residues and a predicted isoelectric point of 9.87. Aside from an
enrichment of basic amino acids at its NH₂ terminus, the acidic and basic residues are distributed throughout the protein. Much of PET100p is predicted to be in an α-helical conformation (Fig. 3). PET100p contains a hydrophobic domain, between amino acid residues 20 and 38. This region is predicted to be composed of two β-strands interrupted by a reverse turn. Based on this predicted structure we propose that this region of the protein is a "hairpin" composed of a self-paired antiparallel β-sheet. The NH₂-terminal domain of PET100p has characteristics of a mitochondrial-targeting sequence. These characteristics include overall hydrophilicity, a high content of basic amino acids, and a predicted α-helical secondary structure. By comparing the NH₂-terminal sequence of PET100p to cleavage site motifs in mitochondrial-targeting peptides (46, 47), we would predict that PET100p falls into the motif that emphasizes the importance of an arginine at position +2, relative to the cleavage site and that the junction between the mitochondrial targeting sequence and mature PET100p is between amino acid residues 13 and 14.

PET100 is located on chromosome IV—During the course of cloning PET100, partial DNA sequences of the ends of clone 2 (Fig. 1) were determined using primers to the polylinker region of pFL38. One of these sequences revealed an open reading frame that corresponds to DGC2, a gene necessary for high-level expression of the pyruvate decarboxylase structural gene (48). PET100 is located on the right arm of chromosome IV.
between PDC2 and the centromere (49) (Fig. 4).

PET100 Is Required for Cytochrome c Oxidase Assembly—The leaky phenotype of the pet100-1 mutant did not allow us to discern whether PET100 is essential for the synthesis or assembly of cytochrome c oxidase. Therefore, a strain carrying a null mutation at the PET100 chromosomal locus (JM43GD100) was constructed by the one-step gene replacement procedure (31). Confirmation that the genomic copy of PET100 is disrupted in JM43GD100 was obtained by comparing genomic Southern blots of strains JM43 and JM43GD100 (Fig. 5). Digestion with the restriction enzymes Apal and BamHI or PstI and BamHI, followed by hybridization with a 1.48-kbp BamHI-PstI probe (Fig. 5A), yielded a single band for JM43GD100 that is 3.0 kbp larger than that produced from JM43 (Fig. 5B) for each set of restriction enzymes. This is the expected result for a PET100 gene carrying a 3-kbp LEU2 insertion and is consistent with the pet100::LEU2 structure shown in Fig. 5A. Moreover, because the Apal site lies outside of the region affected by the gene disruption and because the size of the Apal-BamHI fragment is also increased by 3 kbp in JM43GD100, we conclude that the linear 4.5-kbp BamHI-PstI fragment carrying pet100::LEU2 is integrated into yeast genomic DNA at the PET100 locus and that the integrity of this locus is otherwise unaffected. Finally, no other bands could be detected by hybridization with the 1.48-kbp BamHI-PstI probe, it is unlikely that other copies of this gene exist in the yeast nuclear genome.

To determine the effects of the PET100 null mutation on cytochrome c oxidase, JM43GD100 was compared with the parental strain, JM43, for its ability to grow on nonfermentable medium. From Fig. 6 it is clear that JM43GD100 is unable to grow on medium containing the nonfermentable carbon source glycerol as the sole carbon source. This phenotype for JM43GD100 is more pronounced than that in the pet100-1 mutants, B9-10B or B9-10D. Diploids resulting from a cross between a pet100-1 strain (B9-10D) and the pet100::LEU2 disrupted strain (JM43GD100) were constructed to verify that complementation by the cloned DNA reflects the presence of the wild-type allele of the PET100 gene and not the presence of another gene that could suppress the pet100-1 defect. The growth characteristics of these diploids on YPG medium indicate that the wild-type growth phenotype is not rescued (Fig. 6). This is expected for two mutations that are allelic. Cells from the diploid strain were sporulated and the haploid progeny from eight tetrads were allowed to germinate on YPD medium at 30°C. When grown on glycerol-containing media (YPG), two of the four haploid segregants from each tetrad showed reduced growth, like the pet100-1 mutant, and the other two spores failed to grow altogether, like pet100::LEU2 (data not shown). These latter spores were shown to carry a pet100::LEU2 allele by their ability to grow on leu- media. Together, these results clearly establish that pet100-1 and pet100::LEU2 are allelic and that the growth phenotype of the null mutation is more severe than that of pet100-1.

The effects of the pet100-1 and pet100::LEU2 mutations on growth is paralleled by a decreased specific activity of cytochrome c oxidase in isolated mitochondria (Table II). Disruption of the PET100 gene in JM43GD100 results in the complete abolishment of mitochondrial cytochrome c oxidase activity. In contrast, B9-10D retains 18.6% of the level of cytochrome c oxidase activity present in its respiratory competent parent, JM43. Room temperature cytochrome spectra of mitochondria from JM43, B9-10D, and JM43GD100 reveal that the absorption band corresponding to cytochromes aa3 is reduced in size in strain B9-10D and is completely absent from strain JM43GD100 (Fig. 7). In contrast, the level and absorption maxima for cytochromes c + c2 and b are unaffected in these strains. Low temperature cytochrome spectra (not shown) which allow for partial resolution of cytochromes c and c2 show that the relative levels of these two cytochromes are unaffected in these strains as well. Together, these results indicate that the PET100 gene product is specifically required for cytochrome c oxidase. They also demonstrate that the pet100-1 mutation results in only partial loss of function.

Although the above phenotypic characteristics of JM43GD100 clearly show that PET100 is essential for cytochrome c oxidase, they do not reveal how PET100p functions. The phenotypes of these mutants could result from an effect of PET100p on 1) the synthesis or intracellular localization of subunit polypeptides, 2) the assembly of subunit polypeptides into a functional holoenzyme, or 3) heme A biosynthesis. To distinguish between the first two possibilities, we performed...
Western immunoblot analysis on mitochondrial extracts from mutant and parental strains. For these studies anti-holoenzyme antibodies that recognize all of the polypeptide subunits of cytochrome c oxidase were used. Autoradiograms of Western blots show that all of the cytochrome c oxidase subunits are present in strains B9-10D and JM43GD100 (Fig. 8). These co-migrate with mature subunits from both purified cytochrome c oxidase and the parental strain, JM43. Hence, the subunits appear to be properly processed from their preprotein precursors. The level of some subunits (subunits I, VII + VIIA, VIII) are unaffected in either mutant while the levels of other subunits (subunits II, III, IV, V, and VI) are partially reduced in both mutants. The reduction in levels of the latter subunits is more pronounced in mitochondria from JM43GD100. This reduced steady-state concentration of subunits in the mutant mitochondria may result from an increased rate of subunit turnover. Taken together, these findings make it clear that PET100 is not essential for the synthesis of the subunit polypeptides of cytochrome c oxidase or for their localization to the mitochondrion.

To determine if PET100p is involved in heme A biosynthesis, we tested whether heme A or hemin could rescue the respiration-deficient phenotype of JM43GD100. As controls, we used strains CR7 and CR18 with mutations in HEM2, which encodes δ-aminolevulinate dehydratase (30), and COX11, which is involved in the conversion of protoporphyrin IX to heme A (19). These three strains and JM43 were grown on YPG in the presence of added hemin chloride or heme A. In the absence of added hemin or heme A, JM43GD100 exhibited no growth, while CR7 and CR18 exhibited only slight growth (Fig. 9). In the presence of hemin, CR7 grew as well as JM43, JM43GD100 did not grow, and CR18 grew slightly (data not shown). These are the expected results and are consistent with the observation that hemin can rescue growth of a hem2 mutant on non-fermentable carbon sources (30). Heme A supplementation promoted the growth of CR18 and CR7 but not JM43GD100 (Fig. 9). While this is the expected result for the cox11 mutant, it is somewhat surprising that heme A can rescue a hem2 mutant. However, it is possible that some of the added heme A is degraded to protoporphyrin IX, or some other intermediate in
and strain B9-10D (parental strain JM43) were streaked onto solid YPG medium and incubated at 30 °C for 3 days. The parental strain JM43 (position 1), strain J M43GD100 (position 2), strain B9-10B (position 3), strain B9-10 X JM43GD100 (positions 4 and 5), and strain B9-10D (position 6).

Protoporphyrin IX synthesis, upon entering cells. These findings indicate that heme A can rescue the respiration-deficient phenotype of both a cox11 and hem2 mutant but not a pet100 mutant. Because two strains with different blocks in heme A biosynthesis are rescued by heme A under conditions where heme A does not rescue J M43GD100, it is unlikely that PET100p is required for the biosynthesis of heme A.

**DISCUSSION**

We describe here the isolation and initial characterization of PET100, a gene that is essential for the biogenesis of a functional cytochrome c oxidase complex in the yeast S. cerevisiae. PET100 encodes a polypeptide with a putative NH₂-terminal mitochondrial import sequence as well as a putative membrane insertion sequence and is likely to act late in the assembly of cytochrome c oxidase after subunit polypeptides have been synthesized, processed, and localized to the mitochondrion.

**Chromosome Location and Copy Number of PET100**—During the course of this study an open reading frame corresponding to PET100 was discovered on chromosome IV of S. cerevisiae by the Yeast Genome Sequencing Project (49). The ORF is located on the right arm of chromosome IV between the PDC2 gene and the centromere. Two lines of evidence suggest that PET100 is a unique gene and is not part of a multigene family. First, a null mutant that lacks a functional copy of PET100 completely lacks cytochrome c oxidase. If PET100 were a member of a multigene family it is likely that one of the other members of the family could provide partial function in the pet100 null mutant, as occurs in PET100 mutant but not a PET100::LEU2 null

**FIG. 6.** Growth of strains carrying PET100, pet100-1, and pet100::LEU2 genes on a nonfermentable carbon source. Strains J M43 (PET100), J M43GD100 (pet100::LEU2), B9-10B (pet100-1), B9-10D (pet100-1), and a B9-10 X J M43GD100 diploid strain were streaked onto solid YPG medium and incubated at 30 °C for 3 days. The parental strain J M43 (position 1), strain J M43GD100 (position 2), strain B9-10B (position 3), strain B9-10 X J M43GD100 (positions 4 and 5), and strain B9-10D (position 6).

**FIG. 7.** Absorption spectra of cytochromes from strains carrying PET100, pet100-1, and pet100::LEU2 genes. Cytochrome contents of mitochondria from strains J M43, B9-10D, and J M43GD100 were determined by room temperature difference spectroscopy with an Amino DW-2000 double beam dual wavelength spectrophotometer. An aliquot of isolated mitochondria (5 mg of protein) was suspended in 40 mM KPO₄ (pH 7.4) to 1 ml. The cytochromes were reduced with 15 μl of freshly made 0.5 M sodium dithionite. The spectra of the samples were scanned reading absorbance range 390–700 nm using 577 nm as the reference wavelength with the following settings: dual beam, wavelength acquisition, slit width 3.0 nm. The cytochromes were then oxidized with 20 μl of 0.5 M potassium ferricyanide, and a second spectrum was scanned. The difference spectra were recorded with the second spectrum subtracted from the first. The α absorption bands for cytochromes c + c₁, b, and aa₃ are noted. Top trace = J M43 (PET100); middle trace = B9-10D (pet100-1); bottom trace = J M43GD100 (pet100::LEU2).

**FIG. 8.** Western immunoblot analysis of cytochrome c oxidase from strains carrying PET100, pet100-1, and pet100::LEU2 genes. SDS-solubilized yeast cytochrome c oxidase (1.2 μg of protein) or SDS-solubilized mitochondria (10 μg of protein) were separated on 16% SDS-polyacrylamide gels containing 10% glycerol and 3.6 M urea and transferred to nitrocellulose. The subunits of cytochrome c oxidase were detected by reaction with a mixture of subunit-specific antibodies, followed by incubation with horseradish peroxidase-linked secondary antibodies. The subunits of the enzyme are identified in the margin. Lane 1, purified cytochrome c oxidase; lane 2, J M43 (PET100); lane 3, J M43GD100 (pet100::LEU2); lane 4, B9-10D (pet100-1). The band that appears immediately below subunit VI in lanes 2 and 3 is a contaminating protein of unknown origin that is recognized by one of the antibodies.

**FIG. 9.** The respiration-deficient phenotype of a strain carrying a pet100::LEU2 null mutation is not rescued by added heme A. Strains J M43 (PET100), J M43GD100 (pet100::LEU2), CR7 (hem2), and CR18 (cox11) were streaked onto solid YPG and YPG containing 26 μg/ml heme A and incubated at 30 °C for 4 days. Position 1, J M43; position 2, J M43GD100; position 3, CR7; and position 4, CR18.

**FIG. 10.** The DNA sequence of PET100 predicts a protein product consisting of 111 amino acids. Two aspects of this sequence are of interest from the viewpoint of its role in the assembly of an inner mitochondrial membrane protein. First, the NH₂-terminal 13 amino acids have the characteristics of a mitochondrial-targeting sequence.
Cloning and Characterization of PET100

(46, 47, 50), suggesting that PET100p is a mitochondrial protein. Second, PET100p contains a hydrophobic region that could function in membrane insertion. This region lies between amino acid residues 20 and 38 and is predicted to form a hairpin structure composed of an antiparallel beta-sheet. A similar structure has been predicted to be present in yeast cytochrome c oxidase subunit VIIa (51). These characteristics of PET100p, together with the finding that it affects the assembly process after the subunit polypeptides of cytochrome c oxidase have been localized to the mitochondrion, lead us to the hypothesis that this protein is a mitochondrial membrane protein. Further study is required to confirm this and to determine whether it resides in the inner or outer mitochondrial membrane.

Essentiality and Role of PET100p in Holocytochrome c Oxidase Assembly—The results reported here for a null mutant lacking a functional PET100p gene have allowed us to reach the following conclusions. First, PET100p is essential for the biogenesis of cytochrome c oxidase. Second, PET100p does not have a generalized function in cytochrome biogenesis because other cytochromes (i.e., cytochromes c, c1, and b) are present at normal levels in the null mutant. Third, PET100p does not function in the biosynthesis of heme A. Fourth, because all of the polypeptide subunits of cytochrome c oxidase are present in mitochondria from the null mutant, PET100p is not essential for the synthesis of either the nuclear- or mitochondrially encoded subunits of the complex. This finding also suggests that PET100p is not essential for the import, processing, or localization of the nuclear-coded subunit polypeptides. Although we cannot exclude the possibility that PET100p functions in the submitochondrial localization of one or more of the subunit polypeptides of cytochrome c oxidase, this seems unlikely because the pet100 null mutant does not affect the biogenesis of other cytochromes, and PET100p has no similarity to any known component of the mitochondrial localization machinery (9, 17). All of these conclusions are consistent with the likelihood that PET100p is required for assembly of cytochrome c oxidase.

From its DNA sequence, clearly PET100 does not correspond to any of the structural genes for the nine subunit polypeptides of yeast cytochrome c oxidase. Nor is it identical to COX12 or COX13. Hence, PET100p cannot be considered a subunit of the holozyme. In theory, PET100p could act either 1) directly on assembly itself or 2) indirectly, as a protein that regulates expression of a nuclear gene for a protein that acts on assembly. We believe that the first possibility is more likely, because there is a putative mitochondrial targeting sequence at the NH2 terminus of PET100p. This implies that PET100p functions in the mitochondrion and not in the cytosol or nucleus. Because the PET100 gene exhibits nearly no codon bias (−0.0185), it is likely to be expressed at very low levels (45).

Previous studies have revealed the existence of seven other proteins, COX10p, COX11p, SCO1p, PET117p, PET191p, OXA1p, and COX14p, that are required for the assembly of yeast cytochrome c oxidase but are not themselves subunits of the holozyme (9, 21–23, 52–54). Like PET100p these are specific for cytochrome c oxidase and have no known effects on the assembly of other complexes of the inner mitochondrial membrane. There are no conserved motifs among these proteins, and none of these proteins have sequence similarities with PET100p. So far, three of these proteins, COX11p, SCO1p, and COX14p, have been localized to the membrane fraction from mitochondria (23, 53, 55). It is not yet known, however, whether they reside in the inner or outer membrane. Of these seven proteins the best understood are COX10p and COX11p. These are believed to participate in the heme A biosynthetic pathway. COX10p has been proposed to be a farnesyl transferase that participates in the farnesylation of the vinyl group at carbon 2 of protoheme b, which is a direct precursor to heme A (19). Homologues of the COX10 gene have been found in bacteria (56–59) and in humans (60). COX11p has been proposed to be involved in the paired oxidation/oxygenation step that converts the methyl group at carbon 8 of the heme ring to a formyl group (19).

The functions of SCO1p, PET117p, PET191p, COX14p, and OXA1p are currently unclear. Possible functions for these proteins include roles in heme A biosynthesis or in subunit folding and/or assembly. Also unclear is whether all of these proteins function at the same or different steps in assembly. Interestingly to note is that a fundamental difference exists in the steady-state levels of cytochrome c oxidase subunits in a pet100 null mutant and null mutants in the genes that encode some of these other proteins. In the pet100 null mutant, levels of subunits I, VII, VIIA, and VIII are unaffected, and the levels of the other subunits are only partially reduced. In contrast, in cox10, cox11, sco1, pet117, pet191, andcox14 null mutants, the levels of the mitochondrially encoded subunits are greatly reduced. In addition, the levels of some of the nuclear-encoded subunits are reduced to less than half of their levels in their respiratory competent parent strains. The finding that a pet100 null mutant completely lacks cytochrome c oxidase but has higher steady-state levels of cytochrome c oxidase subunit polypeptides than null mutants in these other genes suggests that it may act later in the assembly process than the previously described proteins. Studies are currently underway to construct a subunit assembly pathway and test this hypothesis.

Acknowledgments—We thank Herve Tetelin and Dr. Andre Goffeau for making a partial sequence of yeast chromosome IV available before publication, Dr. Robin Gutell for advice, Dr. Winslow Caughey for providing purified heme A, and Bradley Goehring for technical assistance.

REFERENCES

1. Trumpower, B. L., and Gennis, R. B. (1994) Annu. Rev. Biochem. 63, 675–716
2. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669
3. Peterson, R. O., Goehring, B., Droste, M., Severson, K. A., Allen, L. A., and Zhao, X. J. (1995) Methods Enzymol. 260, 97–116
4. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995) Science 269, 1069–1074
5. Trueblood, C. E., and Poyton, R. O. (1987) Mol. Cell. Biol. 7, 3520–3526
6. Waterland, R. A., Basu, A., Chance, B., and Poyton, R. O. (1991) J. Biol. Chem. 266, 4180–4186
7. Allen, L. A., Zhao, X. J., Caughey, W., and Poyton, R. O. (1995) J. Biol. Chem. 270, 110–118
8. Patterson, T. E., and Poyton, R. O. (1986) J. Biol. Chem. 261, 17192–17197
9. Poyton, R. O., and McEwen, J. E. (1996) Annu. Rev. Biochem. 65, 563–607
10. Taanman, J. W., and Capaldi, R. A. (1992) J. Biol. Chem. 267, 22481–22485
11. LaMarre, A. E., Albat, M., Chan, S. H., and Trumpower, B. L. (1992) J. Biol. Chem. 267, 22473–22480
12. Taanman, J. W., and Capaldi, R. A. (1993) J. Biol. Chem. 268, 18754–18761
13. McEwen, J. E., Ko, C., Kleoener-Gruissem, B., and Poyton, R. O. (1988) J. Biol. Chem. 263, 11879–11887
14. Tzagoloff, A., and Dieckmann, C. L. (1990) Microbiol. Rev. 54, 211–225
15. Wright, R. M., Trawick, J. D., Trueblood, C. E., Patterson, T. E., and Poyton, R. O. (1987) in Cytochrome Systems: Molecular Biology and Bioenergetics (Papa, S., Chance, B., and Ernst, L., eds) pp. 255–260, Plenum Publishing Co., New York
16. Costanzo, M. C., and Fox, T. D. (1990) Annu. Rev. Genet. 24, 51–113
17. Pon, L., and Schatz, G. (1991) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics (Brockdorff, J., Pringle, J. R., and Jones, E. W., eds) pp. 333–406, Cold Spring Harbor, New York
18. de Wind, J. H., and Grivel, L. A. (1993) Prog. Nucl. Acid Res. Mol. Biol. 46, 51–91
19. Tzagoloff, A., Nobrega, M., Gorman, N., and Sinclair, P. (1993) Biochem. Mol. Biol. Int. 31, 593–598
20. Dieckmann, C. L., and Staples, R. R. (1994) Int. Rev. Cytol. 152, 145–181
21. McEwen, J. E., Hong, K. H., Park, S., and Preciado, G. T. (1993) Curr. Genet. 23, 9–14
22. Bonnefoy, N., Chalvet, F., Hamel, P., Sionisimi, P. P., and Dujardin, G. (1994) J. Mol. Biol. 239, 201–212
23. Gauthier, D. M., Kaelin, T. J., and Tzagoloff, A. (1995) J. Biol. Chem. 270, 15585–15590
