Deletion of the Leader Peptide of the Mitochondrially Encoded Precursor of *Saccharomyces cerevisiae* Cytochrome *c* Oxidase Subunit II

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Manuscript received October 21, 1996
Accepted for publication December 17, 1996

ABSTRACT

Cytochrome *c* oxidase subunit II (Cox2p) of *Saccharomyces cerevisiae* is synthesized within mitochondria as a precursor, pre-Cox2p. The 15-amino acid leader peptide is processed after export to the intermembrane space. Leader peptides are relatively unusual in mitochondrially coded proteins: indeed mammalian Cox2p lacks a leader peptide. We generated two deletions in the *S. cerevisiae COX2* gene, removing either the leader peptide (*cox2-20*) or the leader peptide and processing site (*cox2-21*) without altering either the promoter or the mRNA-specific translational activation site. When inserted into mtDNA, both deletions substantially reduced the steady-state levels of Cox2p and caused a tight nonrespiratory phenotype. A respiring pseudorevertant of the *cox2-20* mutant was heteroplasmic for the original mutant mtDNA and a ρ^- mtDNA whose deletion fused the first 251 codons of the mitochondrial gene encoding cytochrome *b* to the *cox2-20* sequence. The resulting fusion protein was processed to yield functional Cox2p. Thus, the presence of amino-terminal cytochrome *b* sequence bypassed the need for the pre-Cox2p leader peptide. We propose that the pre-Cox2p leader peptide contains a targeting signal necessary for membrane insertion, without which it remains in the matrix and is rapidly degraded.

MOST proteins encoded in mitochondrial DNA (mtDNA) are inserted into the inner membrane where they assemble with nuclearly encoded proteins imported from the cytoplasm (ATTARDI and SCHATZ 1988). The membrane insertion of mitochondrially synthesized proteins, and the export of their hydrophilic domains to the intermembrane space, has not been well characterized, largely due to the lack of a true *in vitro* mitochondrial translation system. Based on experiments with yeast, *in vivo* and in isolated mitochondria, this process is thought to be largely cotranslational (POYTON *et al.* 1992). Membrane bound mRNA-specific translational activators may initiate membrane targeting in yeast mitochondria (FOX 1996), although the possibility of posttranslational insertion has been demonstrated (HERRMANN *et al.* 1995). The machinery responsible for insertion and export of mitochondrially coded proteins has not been identified. However, it does not appear to closely resemble the Sec system of procaryotes since the yeast genome does not contain any close homologues of bacterial *sec* genes that could specify the mitochondrial translocation apparatus (GLICK and VON HEIJNE 1996).

In well characterized procaryotic and eucaryotic systems, exported proteins are targeted to a membrane by signal sequences, often contained in amino-terminal leader peptides that are cleaved off (VON HEIJNE 1988; SCHATZ and DOBBERSTEIN 1996). Leader peptides appear to be rare in mitochondrial systems. There is no evidence for processing of mammalian mitochondrial gene products, and in the cases of mammalian cytochrome oxidase subunit II (Cox2p) and ATP synthase subunit 6, the existence of leader peptides has been ruled out (STEFFENS and BUSE 1979; ANDERSON *et al.* 1982; FEARNLEY and WALKER 1986). Similarly, the *Saccharomyces cerevisiae* mitochondrial gene products cytochrome oxidase subunits I and III, as well as cytochrome *b* and Var1p are known to lack leader peptides (MANNHAUPT *et al.* 1985). However, a few exceptions to this rule have been identified in fungi and plants. In *S. cerevisiae,* both Cox2p (SEVARINO and POYTON 1980; PRATJE *et al.* 1983) and ATP synthase subunit 6 (MICHON *et al.* 1988) are translated with leader peptides, as are *Neurospora crassa* Cox2p (VAN DEN BOOGAART *et al.* 1982) and cytochrome oxidase subunit I (WERNER and BERTRAND 1979). ATP synthase subunit 6 of petunia also has a leader peptide (LU and HANSON 1994).

Both the amino terminal tail and the large acidic carboxy terminal domain of Cox2p are exported from the matrix, through the inner membrane to the intermembrane space (TSUKIHARA *et al.* 1996) (Figure 1A). The *S. cerevisiae* pre-Cox2p leader peptide is cleaved between residues 15 (N) and 16 (D) after transport to the intermembrane space (Figure 1) by an inner membrane bound protease, IMP, that resembles the

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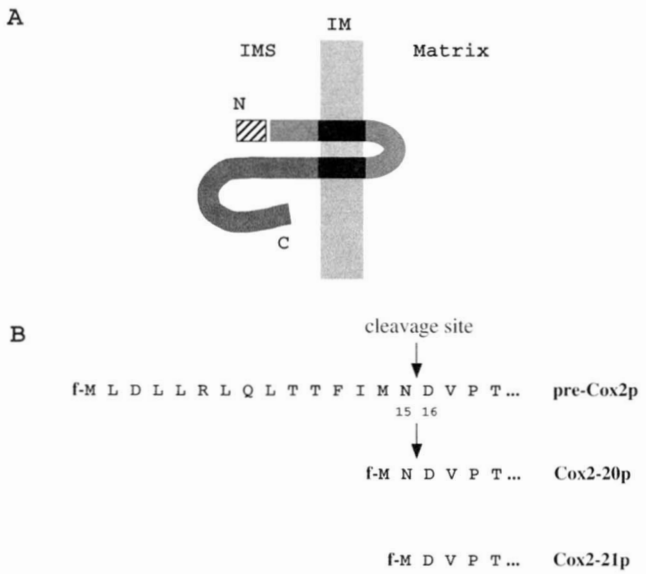

FIGURE 1.—Schematic topology of Cox2p (A), and amino terminal sequences of wild-type pre-Cox2p, and the mutant proteins Cox2-20p and Cox2-21p (B). Both the amino (N) and carboxy (C) termini of Cox2p are in the intermembrane space (IMS). Cox2p has two transmembrane helices spanning the inner membrane (IM); they extend from residues 31 to 61, and from 80 to 107 (■). The leader peptide (▨) is cleaved in the IMS to form mature Cox2p. The proteolytic cleavage site of pre-Cox2p is between residues 15 and 16 of the wild-type protein.

leader peptidase of *Escherichia coli* (PRATJE *et al.* 1983; BEHRENS *et al.* 1991; SCHNEIDER *et al.* 1991; NUNNARI *et al.* 1993). However, the pre-Cox2p leader peptide does not closely resemble bacterial signal sequences (VON HEIJNE 1985; WICKNER *et al.* 1991) as it lacks a hydrophobic core and the third residue is acidic (Figure 1B). Furthermore, cleavage of the leader peptide is not necessary for assembly of active cytochrome *c* oxidase at 23°, since a mutant blocked in this process can grow at that temperature on nonfermentable carbon sources (MANNHAUPT *et al.* 1983). These facts, taken together with the fact that the amino-terminus of mammalian Cox2p is also transported through the inner membrane without a leader peptide (TSUKIHARA *et al.* 1996), raise the question of whether the yeast pre-Cox2p leader peptide has a necessary function in membrane targeting, export or cytochrome oxidase assembly.

In this study we have taken advantage of the fact that *S. cerevisiae* mtDNA can be altered by transformation and homologous gene replacement to delete the pre-Cox2p leader peptide coding sequences *in vivo*. We found that such deletions lower the steady-state level of Cox2p and cause a tight nonrespiratory phenotype. While we were unable to isolate suppressors of a leader peptide deletion that might identify components of the translocation machinery, we found that fusion of the amino-terminal portion of another mitochondrial gene product, cytochrome *b*, to the leaderless Cox2p produced a functional polypeptide that was processed normally. These findings demonstrate the importance of the pre-Cox2p leader peptide, probably in membrane targeting, and suggest that the unprocessed amino-termini of other mitochondrial gene products can carry out similar functions.

MATERIALS AND METHODS

Strains, media and genetic methods: The *S. cerevisiae* strains used in this investigation are listed in Table 1. Media and mitochondrial genetic manipulations were as described (FOX *et al.* 1991; MULERO and FOX 1993a). Standard genetic methods were as described (ROSE *et al.* 1988).

Construction of the *cox2-20* and *cox2-21* deletion mutations by *in vitro* oligonucleotide-directed mutagenesis (Mutagene Kit, Bio-Rad) was performed as previously described using the plasmid pJM2 as a template (MULERO and FOX 1993a). *cox2-20* was generated with the oligonucleotide 5′-GTGTTGGTACATCATTCATTTTAATAAATCTTAAC, complementary to the *COX2* mRNA sequence at positions −16 to +3 and +43 to +58, with respect to the translation start codon. *cox2-21* was generated with the oligonucleotide 5′-AAGGTGTTGGTACATCCATTTTAATAAATCTTAAC, complementary to the COX2 mRNA sequence at positions −16 to +3 and +46 to +61. Accurate mutagenesis was confirmed by DNA sequence analysis.

ρ^0 mitochondria of strain MCC109ρ^0 were transformed with the mutagenized plasmids by microprojectile bombardment as previously described (FOX *et al.* 1991; MULERO and FOX 1993a) and mitochondrial transformants were identified by their ability to produce respiring diploids when crossed to the ρ^+, *cox2* tester strain VC32.

Detection of Cox2p by immunoblotting: Total yeast proteins were extracted as described (YAFFE 1991) from cells grown to saturation in rich medium containing either ethanol and glycerol (YPEG), or galactose (YPGal) as indicated. Proteins were separated by SDS-PAGE (12% acrylamide), and analyzed by Western blotting under standard procedures (SAMBROOK *et al.* 1989), using the anti-Cox2p mouse monoclonal antibody CC06 (a gift from THOMAS L. MASON). CC06 was used at a 1/50 dilution and the secondary anti-mouse antibody (Gibco BRL, Inc.) at a 1/5000 dilution. Binding of the secondary antibody was revealed using the ECL detection kit of Amersham.

DNA and RNA isolation, electrophoresis, and hybridization: Genomic DNA was isolated as described (HOFFMAN and WINSTON 1987). *Hpa*II-digested DNA was separated by electrophoresis on a 1.5% agarose gel, transferred to a nylon membrane and crosslinked under UV light. High-stringency hybridization at 65° was as described (SAMBROOK *et al.* 1989). Cellular RNA was isolated as described (ROSE *et al.* 1988) from cells grown in rich galactose medium (YPGal). Five micrograms of each sample were electrophoresed in a 1.4% agarose gel containing formaldehyde, blotted to nitrocellulose and hybridized as described (SAMBROOK *et al.* 1989). The *COX2*-specific probe used for hybridization analyses was ^{32}P-labeled by PCR amplification (INNIS and GELFAND 1990) of the entire *COX2* gene from total DNA of strain DL1 using primers termed "a" and "d" in the legend to Figure 6 (see below), in the presence of dATP, dGTP, dTTP and α-^{32}P-dCTP.

DNA sequence and PCR analysis: mtDNA templates for DNA sequence analysis were prepared by CsCl centrifugation as described (FOX *et al.* 1991). Sequences were determined by extending the *COX2* specific primer 5′-CATTCAAGATACTAAACCTAAAATAAC (termed "b" in the legend to Figure 6) using the dsDNA Cycle Sequencing kit from Gibco BRL, Inc. PCR was carried out as follows: 94° 1 min; 25 cycles, 94° 30 sec, 55°

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
DAU1ρ^0	*MAT*α ade2 ura3 [ρ^0]	This study
DL1	*MAT*α lys2 [ρ^+]	FOLLEY and FOX (1991)
DL2	*MAT***a** lys2 [ρ^+]	FOLLEY and FOX (1991)
DS302	*MAT*α met [ρ^- COX2]	CORUZZI *et al.* (1981)
MCC109ρ^0	*MAT*α ade2-101 ura3-52 kar1-1 [ρ^0]	COSTANZO and FOX (1993)
TF187	*MAT***a** his3-11,15 leu2-3,112 [ρ^+ cox2-17]	This study
VC32	*MAT***a** his4 pet9 [ρ^+ cox2]	WILSON and CAMERON (1994)
VC200	*MAT*α ade2-101 ura3-52 kar1-1 [ρ^- cox2-20]	This study
VC203Δ	*MAT***a** lys2 [ρ^+ cox2-20]	This study
VC203ΔR	*MAT***a** lys2 [ρ^+ cox2-20; ρ^- cob::cox2-20]	This study
VC204	*MAT*α ade2 ura3 [ρ^- cob::cox2-20]	This study
VC300	*MAT*α ade2-101 ura3-52 kar1-1 [ρ^- cox2-21]	This study
VC303Δ	*MAT***a** lys2 [ρ^+ cox2-21]	This study

1 min, 65° 1 min; 65° 10 min. Additional primers employed in the experiment of Figure 6 were as follows:"a," 5′-GACAAAAGAGTCTAAAGGTTAAGA; "c," 5′-TGAACAATTTTTCCAGCTGTAATT; "d," 5′-ATAAAAACTACCATCTCCATCTGT; "cob," 5′-GGACAGATGTCACATTGAGGTGC.

RESULTS

Deletions in mtDNA that eliminate the leader peptide of pre-Cox2p: To initiate studies on the function of the pre-Cox2p leader peptide, we generated two deletion mutations *in vitro* that removed most or all of its coding sequence (MATERIALS AND METHODS). The *cox2-20* allele removed 13 codons, fusing the translation initiation site to codon 15. The polypeptide coded by *cox2-20* contains the peptide bond between residues 15 and 16 that is normally cleaved during maturation of Cox2p (Figure 1B). The second mutation, *cox2-21*, deleted 14 codons, fusing the initiation site to codon 16, thus eliminating the cleavage site (Figure 1B). We made two mutations to enhance our chances of generating a functional protein. Plasmids carrying these mutant alleles were introduced separately into the mitochondrial compartment of a ρ^0 (lacking mtDNA) recipient by microprojectile bombardment and the resulting synthetic ρ^- transformants were isolated (MATERIALS AND METHODS). The plasmid-borne wild-type *COX2* gene can support respiratory growth in *trans* in heteroplasmic strains formed by mating a synthetic ρ^- transformant to a ρ^+, *cox2* deletion mutant (FOX *et al.* 1988; MULERO and FOX 1993a). However, heteroplasmic strains generated by mating synthetic ρ- transformants carrying either *cox2-20* or *cox2-21* to the ρ^+, *cox2* deletion mutant TF187 failed to grow on nonfermentable (YPEG) medium, demonstrating that both mutations prevented *COX2* function.

Homoplasmic ρ^+ strains carrying each of the leader peptide mutations integrated into mtDNA were generated by replacing the wild-type gene with mutant alleles by homologous recombination (FOLLEY and FOX 1991; FOX *et al.* 1991). The *kar1-1* (CONDE and FINK 1976) synthetic ρ^- transformants were mated with the ρ^+ wild-type strain DL2 to allow homologous recombination of mtDNAs and the production of haploid cytoductants. Nonrespiring haploid cytoductants, VC203Δ and VC303Δ (Table 1), with the *cox2-20* and *cox2-21* mutations, respectively, integrated in ρ^+ mtDNA were identified by their ability to produce respiring diploids when mated to a ρ^-, *COX2* tester strain (DS302).

Phenotypic characterization of leader peptide deletion mutants: The deletion mutations affecting the pre-Cox2p leader peptide did not affect the *COX2* promoter sequence (BORDONNÉ *et al.* 1988; CAMERON *et al.* 1989), but could have altered mRNA stability. To determine if *cox2-20* and *cox2-21* interfered with gene expression at the level of mRNA accumulation, we carried out RNA-gel-blot hybridization analysis on RNA isolated from wild-type and mutant strains (Figure 2). Both of the leader peptide mutants contained approximately wild-type levels of the major 875 base *COX2* mRNA, slightly shortened in the mutants. In addition, they contained the secondary *COX2* transcript of roughly 2400 bases, which has the same 5′-end as the major mRNA (BORDONNÉ *et al.* 1988), as well as other less abundant transcripts of the region. Thus, these mutations do not block expression at the level of mRNA accumulation.

The *cox2-20* and *cox2-21* mRNAs should be translated normally since they bear the wild-type *COX2* 5′-untranslated leader and translation initiation codon. This 5′-untranslated leader contains the mRNA-specific translational activation site that directs translation of Cox2p and of other protein coding sequences placed downstream of it (MULERO and FOX 1993b; D. F. STEELE, H. M. DUNSTAN and T. D. FOX, unpublished results). To test whether the protein products of the *cox2* mutant alleles accumulate in mitochondria, we analyzed total cell protein extracts by immunoblotting using an anti-Cox2p mouse monoclonal antibody provided by T. L. MASON (Figure 3). Cox2p was easily detectable in wild-

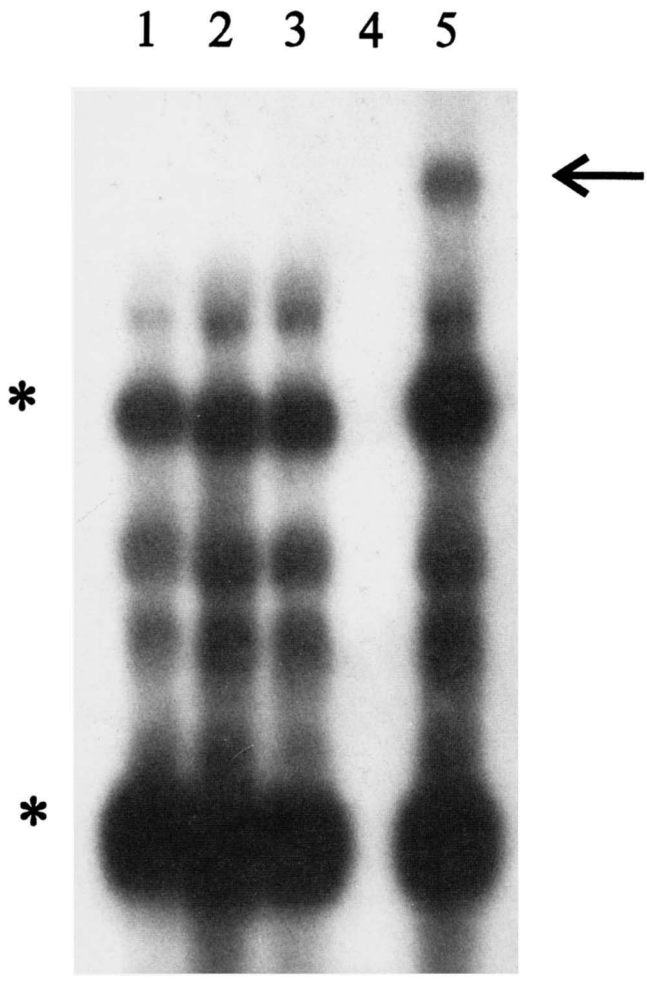

FIGURE 2.—*COX2* mRNA levels are unaffected by the *cox2-20* and *cox2-21* mutations. Total cellular RNA was prepared and analyzed by RNA-gel-blot hybridization with a *COX2*-specific probe (MATERIALS AND METHODS). Lane 1, wild-type DL1; lane 2, the *cox2-20* mutant VC203Δ; lane 3, the *cox2-21* mutant VC303Δ; lane 4, the *cox2* deletion mutant TF187; lane 5, the pseudorevertant VC203ΔR. * mark the major *COX2* transcripts of 875 and 2400 bases. The arrow marks a novel *cob-cox2* transcript (see text).

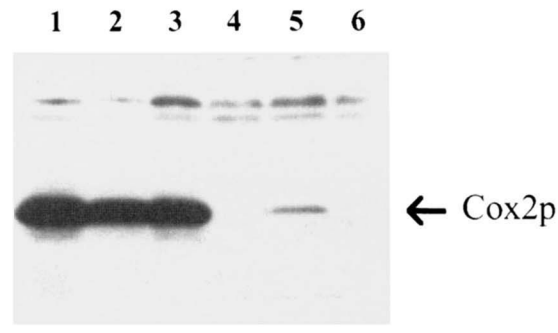

FIGURE 3.—Immunodetection of Cox2p in wild-type and mutant strains. One hundred micrograms of total yeast proteins were loaded in each lane and reacted with an antibody directed against Cox2p (MATERIALS AND METHODS). The band corresponding to Cox2p is indicated. Cells for the extracts in lanes 1 and 2 were grown in nonfermentable medium (YPEG), whereas cells for the extracts in lanes 3–6 were grown in galactose medium (YPGal). Lanes 1 and 3, the wild-type DL2; lane 2, the pseudorevertant VC203ΔR; lane 4, the *cox2* deletion mutant TF187; lane 5, the *cox2-20* mutant VC203Δ; lane 6, the *cox2-21* mutant VC303Δ.

type extracts (Figure 3, lane 3) but absent in an extract of a strain bearing a complete *cox2* deletion (Figure 3, lane 4). The *cox2-20* mutant, which retains the cleavage site of pre-Cox2p, contained greatly diminished levels of Cox2p (Figure 3, lane 5). The *cox2-21* mutant, which lacks the cleavage site, contained even less Cox2p (Figure 3, lane 6): upon longer exposure a faint Cox2p band was observed in the *cox2-21* mutant extract but not in the extract of the complete *cox2* deletion strain (not shown). Thus, deletion of the pre-Cox2p leader peptide reduces the steady-state level of Cox2p, probably by reducing stability of the protein.

Isolation of a *cox2-20* pseudorevertant: In *E. coli*, secretion defects caused by the absence of a leader peptide can be suppressed by mutations affecting the translocation machinery (DERMAN *et al.* 1993). In an effort to identify analogous suppressors in the mitochondrial system, we selected for respiring (Pet^+) revertants of the *cox2-20* strain VC203Δ. However, we were unable to isolate respiring revertants in preliminary screens, either spontaneously or with UV mutagenesis.

We obtained a single spontaneous respiring revertant of VC203Δ from a large-scale experiment in which $\sim 10^{11}$ cells, grown in 1 liter of glucose containing medium, were plated on nonfermentable medium (YPEG). This revertant, VC203ΔR, exhibited a mitotically unstable Pet^+ phenotype upon repeated restreaking, similar to previously characterized strains that require two different mtDNAs for respiratory growth (MÜLLER *et al.* 1984). Such mitochondrially heteroplasmic strains typically contain the complete ρ^+ genome of the original mutant and a ρ^- mtDNA bearing a gene rearranged so as to allow expression. Mitotic instability results from the rapid segregation of distinct mtDNAs from each other at cell division.

To confirm the existence of a mitochondrial genetic element suppressing *cox2-20,* VC203ΔR was crossed to a ρ^0 strain (DAU1ρ^0) and the resulting Pet^+ diploid was sporulated. Tetrads were dissected on complete glucose medium (YPD). While the nuclear markers segregated 2:2, all the spore clones were Pet^-, as expected if two mtDNAs were segregating during growth on nonselective medium. However, when mated back to ρ^+ strains carrying the *cox2-20* mutation, most of the haploid progeny (seven out of eight tested) produced mitotically unstable Pet^+ diploids, indicating that they contained a suppressing mtDNA.

Suppression of the cox2-20 deletion by fusion of cytochrome *b* (*COB*) coding sequence to its 5′-end: We detected both rearranged *COX2* sequences and the original *cox2-20* gene in the pseudorevertant VC203ΔR by DNA-gel-blot hybridization analysis of genomic DNA cleaved with *Hpa*II. *Hpa*II generates a 2.4-kb fragment

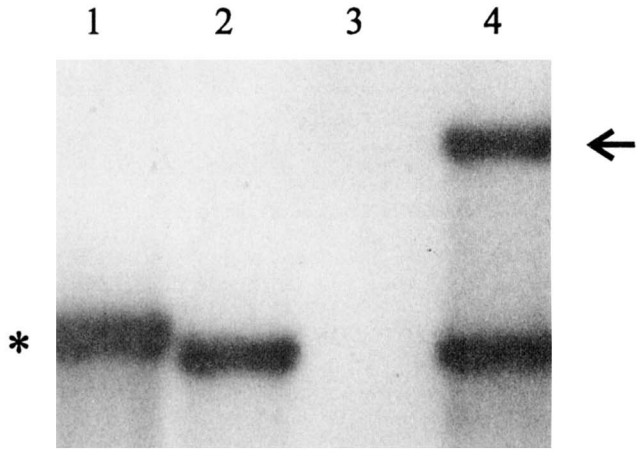

FIGURE 4.—VC203ΔR contains both rearranged *COX2* sequences and the original *cox2-20* gene. Total cellular DNA was prepared, digested with *Hpa*II and analyzed by gel-blot-hybridization with a *COX2*-specific probe (MATERIALS AND METHODS). * indicates the 2.4-kb wild-type fragment and the arrow indicates the novel rearranged fragment. Lane 1, the wild-type DL1; lane 2, the *cox2-20* mutant VC203Δ; lane 3, the *cox2* deletion mutant TF187; lane 4, the pseudorevertant VC203ΔR.

of wild-type mtDNA containing the entire *COX2* gene (FOX 1979b) (Figure 4, lane 1). A slightly shorter *COX2* fragment was observed in DNA of both the VC203Δ mutant (Figure 4, lane 2) and of the VC203ΔR pseudorevertant (Figure 4, lane 4). In addition, the pseudorevertant DNA contained a novel hybridizing fragment of ~2.9 kb.

Sequence analysis of mitochondrial DNA isolated from a haploid containing only the suppressor genome (VC204) revealed that it is a *cob::cox2-20* translational fusion between *COB* codon 251 (in the first exon of strain D273-10B) and *COX2* codon 15 (Figure 5). Such a fusion should produce a 2.9-kb *Hpa*II fragment, like that observed in Figure 4, based on the known positions of the *Hpa*II sites in the *COB* (NOBREGA and TZAGOLOFF 1980) and *COX2* (CORUZZI and TZAGOLOFF 1979; FOX 1979a) regions of mtDNA.

To confirm the structure of the chimeric gene, and the heteroplasmic nature of the pseudorevertant VC203ΔR, we performed PCR amplification of wild-type, mutant and pseudorevertant DNA templates using *COB*- and *COX2*-specific primer pairs (Figure 6). The expected *COB-COX2* chimera-specific fragment could be amplified only from DNA of the heteroplasmic pseudorevertant. This PCR product was sequenced and shown to contain the *cob::cox2-20* fusion (not shown). *COX2*-specific primer pairs amplified the expected fragments corresponding to unrearranged mtDNA from all three templates, confirming that VC203ΔR is heteroplasmic, and demonstrating that no downstream rearrangements of the *COX2* gene had occurred.

Hybridization analysis of RNA from the heteroplasmic pseudorevertant VC203ΔR indicated that the *cob::cox2-20* chimeric gene is transcribed. This strain contains a novel transcript of roughly 4 kb that is absent from the wild-type and mutant strains (Figure 2, lane 5). The size of this new RNA is compatible with the predicted size of a chimeric gene transcript, having the 5′-end of the *COB* mRNA followed by 950 bases of *COB* 5′-untranslated leader (DIECKMANN and STAPLES 1994) and 753 bases of *COB* coding sequence, plus 2300 bases of the secondary *COX2* transcript (the *cob::cob2-20* fusion removes about 100 bases of *COX2* sequence). Joining of these same *COB* sequences to the smaller major *COX2* mRNA would produce an RNA of 2400 bases, indistinguishable in this experiment from the wild-type secondary *COX2* transcript.

Expression of the *cob::cox2-20* fusion gene leads to accumulation of apparently normal Cox2p: A protein product of the *cob::cox2-20* chimeric mRNA in the heteroplasmic pseudorevertant must be functional, since the strain can grow on nonfermentable carbon sources. The initial translation product should be a polypeptide roughly twice the size of mature wild-type Cox2p. However, while this polypeptide lacks the leader peptide sequence of pre-Cox2p, it should have the amino-terminal sequence of cytochrome *b* and contain the peptide bond that is normally cleaved to generate mature Cox2p, between wild-type residues 15 and 16 (Figure 7). The chimeric cytochrome *b*-Cox2p fusion protein is apparently cleaved at this site, since the pseudorevertant contains Cox2p that is indistinguishable in size from wild type (Figure 3, lane 2).

DISCUSSION

In this study, we generated two deletion mutations in the *COX2* mitochondrial gene that shorten the 15-

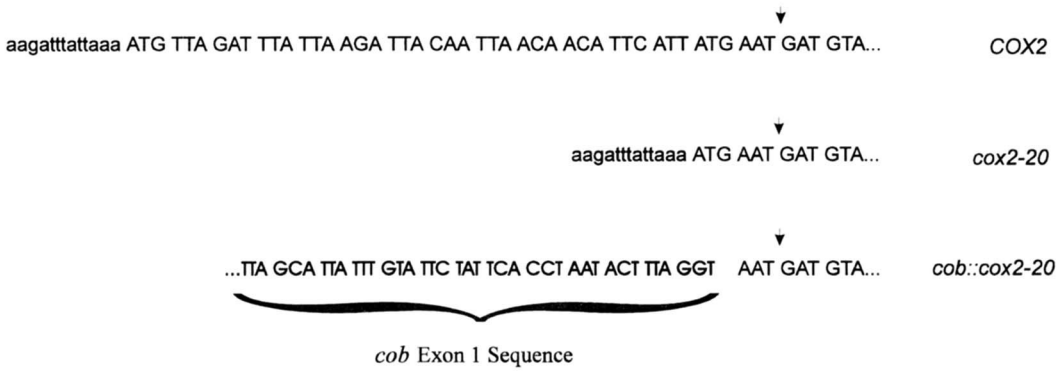

FIGURE 5.—DNA sequences of *COX2*, *cox2-20* and the *cob::cox2-20* chimeric gene. The arrows indicate the position of the proteolytic cleavage site in the encoded proteins.

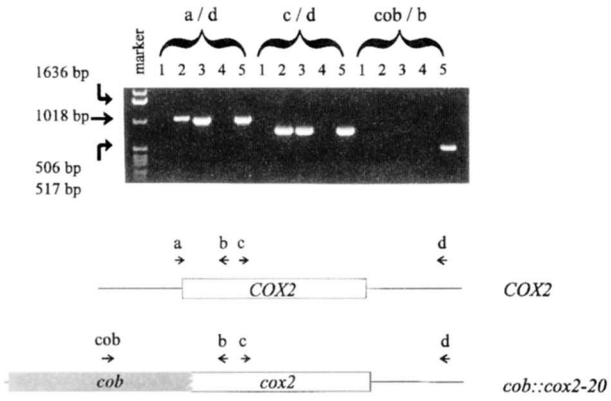

FIGURE 6.—PCR amplification of *COX2*, *cox2-20* and *cob::cox2-20* sequences from wild-type mutant and pseudorevertant strains. Total DNA was isolated from the wild-type strain DL1, the *cox2-20* mutant strain VC203Δ, the *cox2* deletion mutant TF187, and the heteroplasmic revertant VC203ΔR. The PCR was carried out with the indicated primer pairs (MATERIALS AND METHODS) and the products analyzed by agarose gel electrophoresis. The expected fragment sizes for each combination of primer pair and template are as follows: pair a/d on *COX2* mtDNA, 1069 bp; pair a/d on *cox2-20* mtDNA, 1030; pair a/d on *cob::cox2* mtDNA, no product; pair c/d on all templates, 783 bp; pair cob/b on *COX2* and *cox2-20* mtDNA, no product; pair cob/b on *cob::cox2* mtDNA, 520. Lanes 1, no DNA; lanes 2, the wild-type DL1; lanes 3, the *cox2-20* mutant VC203Δ; lanes 4, the *cox2* deletion mutant TF187; lanes 5, the pseudorevertant VC203ΔR.

FIGURE 7.—Amino acid sequences of three proteins cleaved in the intermembrane space by the Imp1p protease. Sequences derived from Cox2p are underlined.

amino acid pre-Cox2p leader peptide by 13 or 14 amino acids. In one deletion, the cleavage site between residues 15 and 16 was retained while in the other it was not. Both deletions substantially reduced the steady-state levels of Cox2p and caused a strong nonrespiratory phenotype, indicating that the leader peptide has an important function in yeast.

The leader peptide deletion mutations appear to block *COX2* gene expression at a posttranslational level. *COX2* mRNA steady-state levels were unaffected by the deletions. Furthermore, the mutant mRNAs both carried the complete wild-type 5′-untranslated leader, which contains a site for activation of translation by the mRNA-specific activator protein Pet111p (POUTRE and FOX 1987; MULERO and FOX 1993a,b). Previous work from this laboratory strongly suggests that there are no sequences required for translation in the protein coding region of the *COX2* gene. Placement of the *COX2* mRNA 5′-untranslated leader upstream of either the endogenous *COX3* structural gene or the synthetic *ARG8*^{m} structural gene (STEELE *et al.* 1996) is sufficient to activate translation of those genes (MULERO and FOX 1993b; D. F. STEELE, H. M. DUNSTAN and T. D. FOX, unpublished results). Thus it is highly unlikely that the leader peptide deletions interfere with translation.

Our findings are consistent with the hypothesis that leader peptide deletions prevent membrane insertion of pre-Cox2p, or export of its intermembrane space domains, because they remove a targeting signal necessary to initiate translocation. In this view, the mutant polypeptide would remain aberrantly on the matrix side of the inner membrane and be rapidly degraded. Translocated but unassembled Cox2p is degraded in yeast by an ATP-dependent protease of the AAA family encoded by the *YME1* gene (NAKAI *et al.* 1995; PEARCE and SHERMAN 1995; WEBER *et al.* 1996), whose catalytic activity is present on the outer face of the inner membrane (LEONHARD *et al.* 1996). Consistent with our hypothesis, we found that *yme1* disruption had no effect on the steady-state levels of Cox2p in leader peptide deletion mutants (not shown). Untranslocated mutant forms of Cox2p might be degraded by any one or more of several mitochondrially located proteases. These include the Yta10p/Yta12p (Afg3p/Rca1p) ATP-dependent protease of the AAA family located on the matrix side of the inner membrane (TZAGOLOFF *et al.* 1994; GUÉLIN *et al.* 1996; LEONHARD *et al.* 1996) and matrix localized ATP-dependent proteases homologous to bacterial Clp (LEONHARDT *et al.* 1993) and Lon (SUZUKI *et al.* 1994; VAN DYCK *et al.* 1994) proteases.

Mutations in the *E. coli* gene *secY*, which encodes an integral membrane component of the export machinery, can suppress complete signal sequence deletions affecting at least two exported proteins (DERMAN *et al.* 1993). We have so far been unable to select any such suppressors of *cox2* leader peptide deletions. However, we did isolate a single pseudorevertant of the leader peptide deletion, *cox2-20,* that retains the pre-Cox2p cleavage site. In this case, a large ρ^- deletion in mtDNA fused the first 251 codons of the mitochondrial gene encoding the integral membrane protein cytochrome *b* to the *cox2-20* sequence. The proteolytic cleavage site between pre-Cox2p amino acids 15 and 16 remains intact in the predicted fusion protein (Figure 7). When present heteroplasmically with the original ρ^+ mutant mtDNA, this *cob::cox2-20* chimeric gene was expressed allowing respiratory growth and accumulation of Cox2p, indistinguishable in size from wild type. Thus, the presence of amino-terminal cytochrome *b* sequence bypassed the need for the pre-Cox2p leader peptide.

While the amino terminus of yeast cytochrome *b* is not proteolytically processed (MANNHAUPT *et al.* 1985), it appears to contain a targeting signal that can direct Cox2p into the inner membrane. Presumably, mammalian Cox2p contains a similar unprocessed signal. Interestingly, cytochrome *b* residue 251 is thought to be in

a loop exposed in the intermembrane space (TRUMPOWER 1990; GAVEL and VON HEIJNE 1992). Thus the chimeric protein would be expected to deliver the pre-Cox2p cleavage site to the processing protease located on the *trans* side of the membrane. The fact that Cox2p in the pseudorevertant was indistinguishable in size from wild type indicates that proteolytic processing had occurred.

A comparison of known and presumed fungal pre-Cox2p leader peptide sequences (HARDY and CLARK-WALKER 1990) revealed no conservation of charged residues present in the *S. cerevisiae* protein, although the placement of hydrophobic residues is generally similar. Our data do not elucidate the possible role of these hydrophobic residues in targeting since we were only able to suppress the loss of the leader peptide with a gene fusion that bypassed this function.

The Imp1p subunit of the yeast mitochondrial inner membrane protease processes both the mitochondrially coded pre-Cox2p and the nuclearly coded cytochrome b_2 precursor (PRATJE and GUIARD 1986; BEHRENS *et al.* 1991; SCHNEIDER *et al.* 1991; NUNNARI *et al.* 1993). The fusion protein coded by the *cob::cox2-20* gene is cleaved to the size of mature Cox2p, and thus is probably also an Imp1p substrate. There is little similarity among the processing sites of these three proteins. The common features are cleavage of the peptide bond between N and an acidic residue (D or E), and a hydrophobic residue three positions upstream. More refined alterations of pre-Cox2p will be necessary to reveal features of the leader peptide necessary for both targeting and processing.

N.B. was supported by a postdoctoral fellowship from the Human Frontier Science Program Organization. This work was supported by a grant (GM-29362) to T.D.F. from the National Institutes of Health and by a grant (MCB-9317071) to V.L.C. from the National Science Foundation.

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Communicating editor: M. JOHNSTON