A Point Mutation in the Mitochondrial Cytochrome b Gene Obviates the Requirement for the Nuclear Encoded Core Protein 2 Subunit in the Cytochrome bc1 Complex in Saccharomyces cerevisiae*

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A yeast mutant (cor2–45) in which approximately half of the C terminus of core protein 2 of the cytochrome bc1 complex is lacking due to a frameshift mutation that introduces a stop at codon 197 in the COR2 gene fails to assemble the cytochrome bc1 complex and does not grow on non-fermentable carbon sources that require respiration. The loss of respiration is more severe with this frameshift mutation than with the complete deletion of the COR2 gene, suggesting deleterious effects of the truncated core 2 protein. A search for extragenic suppressors of the nuclear cor2–45 mutation resulted (in addition to the expected nuclear suppressors) in the isolation of a suppressor mutation in the mitochondrial DNA that replaces serine 223 by proline in cytochrome b.

Assembly of the cytochrome bc1 complex and the respiratory deficient phenotype of the cor2–45 mutant are restored by the proline for serine replacement in cytochrome b. Surprisingly, this amino acid replacement in cytochrome b corrects not only the phenotype resulting from the cor2–45 frameshift mutation, but it also obviates the need for core protein 2 in the cytochrome bc1 complex since it alleviates the respiratory deficiency resulting from the complete deletion of the COR2 gene. This is the first report of a homoplasmic missense point mutation of the mitochondrial DNA acting as a functional suppressor of a mutation located in a nuclear gene and the first demonstration that the supernumerary core protein 2 subunit is not essential for the electron transfer and energy transducing functions of the mitochondrial cytochrome bc1 complex.

In the mitochondrial respiratory chain, the cytochrome bc1 complex transfers electrons from ubiquinol to cytochrome c and couples this electron transfer to vectorial proton translocation across the inner mitochondrial membrane (for reviews, see Refs. 1, 2). The structure of this respiratory enzyme is best characterized for yeast, beef, and potato where it consists of 10 or 11 different polypeptide subunits, three of which (cytochrome b, cytochrome c1, and the Rieske iron-sulfur protein) carry redox prosthetic groups, and 7 to 8 polypeptides, which lack redox prosthetic groups (core proteins 1 and 2 and five to six small proteins with molecular masses below 15 kDa). The cytochrome b subunit is encoded by the mitochondrial DNA while all the other subunits of the complex are encoded in the nucleus.

The three subunits carrying redox prosthetic groups have been studied extensively, and their roles in electron transfer and proton translocation have been elucidated. Much less is known about the subunits that do not carry redox prosthetic groups. These are often referred to as supernumerary subunits because they have no counterparts in bacteria such as Paracoccus denitrificans and Rhodospirillum rubrum, where the cytochrome bc1 complex contains only cytochrome b, cytochrome c1, and the Rieske iron-sulfur protein (3, 4). The bacterial and mitochondrial cytochrome bc1 complexes exhibit essentially identical electron transfer and proton translocating activities, suggesting that the supernumerary polypeptides of the mitochondrial enzyme are not directly involved in the energy transducing mechanism (5).

This view has received support with the discovery that the core proteins of the Neurospora crassa and plant cytochrome bc1 complexes are MPPs,\(^1\) the proteases that process the precursors of a number of nuclear encoded precursors of mitochondrial proteins (6–10; see Refs. 11–13 for reviews). In yeast α-MPP and β-MPP are water-soluble proteins located in the matrix and not membrane bound (14–16). Although they are proteolytically inactive, the two core proteins of the yeast cytochrome bc1 complex show structural similarities to MPP (17, 18). Thus, it is believed that the core proteins and MPP have a common phylogenetic origin in an ancestral protease and that the proteolytic activity became detached from the yeast complex after some gene duplication occurred (6, 12).

The question which remains to be addressed is what are the functions of the proteolytically inactive core proteins of the yeast cytochrome bc1 complex? Yeast mutants with a structural deficiency in either core protein 1 or core protein 2 fail to properly assemble the cytochrome bc1 complex and, consequently, they do not grow or have extremely reduced growth rates on non-fermentable substrates (17, 18). Here we aimed to know if the respiratory capacity can be restored in such a mutant by a second mutation in another gene, thus allowing the assembly of bc1 complex despite a core protein deficiency.

We first describe a mutant in which about half of core protein

\(^{1}\) The abbreviations used are: MPP, matrix processing protease; PCR, polymerase chain reaction.
2 is lacking due to a frameshift mutation in its gene and show that the assembly of the \textit{bc} \textsubscript{1} complex is blocked in this mutant. We then show that the assembly and activity of the complex are restored by a second mutation located in the mitochondrial DNA which replaces serine 223 by proline in cytochrome \textit{b} and that this single missense mutation eliminates the need for core protein 2. This is the first report of a homoplasmic missense point mutation of the mitochondrial DNA acting as a functional suppressor of a mutation located in a nuclear gene. This also unequivocally establishes that core protein 2 is not centrally involved in the electron transfer and energy transducing functions of the \textit{bc} \textsubscript{1} complex and emphasizes the question of its biological role.

**EXPERIMENTAL PROCEDURES**

Strains and Standard Genetics Methods—The genotypes and origins of the strains used in this study are listed in Table I. Procedures for MnCl\textsubscript{2} mutagenesis (19), random sporation and synchronous crosses (20), cytoduction (21), and induction of \textit{rho}\textsuperscript{0}/\textit{rho} \textsuperscript{-} cells (22) were described previously.

Isolation and Sequence Determination of the Mutant CW30/SM45—The mutant SM45 was selected as a respiratory deficient mutant after MnCl\textsubscript{2} mutagenesis of the wild-type strain CW30 and shown to be a nuclear mutation (see "Results"). Restriction fragment length polymorphism (RFLP) analysis of the mitochondrial DNA from the mutant CW30 and strain CW30 was performed. The 

Enzyme Assays and Western Analysis—Mitochondrial membranes were isolated using a procedure described previously (29). Yeast were grown overnight on 2% glucose, 1% yeast extract, 1% bacopentope, and 2% agar. The cells were harvested by centrifugation at 4,000 \textit{g} for 10 min. They were then washed once with distilled water and resuspended in Tris/mannitol, 50 mM Tris, pH 7.6, and 220 mOsm/kg yeast were disrupted using glass beads in five 1-min intervals using a Becton Products Bead-Beater. The cell extract was diluted with 1 volume of ice-cold Tris/mannitol buffer and centrifuged for 10 min at 1,000 \textit{g} at 4 °C to remove intact cells. The supernatant was saved and recentrifuged at 1,000 \textit{g} for 10 min. Mitochondrial membranes were collected by centrifugation of the supernatant at 10,000 \textit{g} for 20 min. Membranes were washed twice with 150 mM NaCl and recentrifuged. Final membrane pellets were resuspended in 50 mM Tris, 1 mM MgSO\textsubscript{4}, pH 7.6, containing 50% glycerol and stored at −20 °C. Protein concentrations were determined by the method of Lowry et al. (50) as modified by Markwell et al. (51).

Ubiquinol-cytochrome c oxidoreductase activity was assayed at pH 7.0 and 28 °C using the substrate analog 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinol (52). The nonenzymatic rate of cytochrome c reduction was then initiated by adding the membrane fraction to the assay buffer and allowing the reaction to proceed for approximately 5 s. The enzymatic rate was then initiated by adding the membrane fraction to the assay buffer. Rates were obtained in duplicate and varied less than 10%. The amounts of proteins used in the enzyme assays are given in the legend of Fig. 2.

Immunoblot analysis on the mitochondrial membranes prepared as described above was performed with polyclonal antibodies raised against purified cytochrome \textit{bc} \textsubscript{1} complex as described previously (29). The amounts of protein used in the immunoblot analysis are given in the legend of Fig. 4.

**RESULTS**

The Respiratory Deficient Mutant SM45 Results from a Frameshift in the Nuclear Gene Coding for the Core Protein 2 Subunit of the Cytochrome \textit{bc} Complex—The mutant CW30/SM45 (abbreviated SM45; Table I) exhibits a substrate-conditional phenotype commonly observed in strains with respiratory defective mitochondria. It grows on glucose but cannot grow on non-fermentable carbon sources such as glycerol, ethanol, or lactate, the metabolism of which needs functional mit\textit{a} complex (Fig. 1). The phenotype of this mutant was complemented by crossing with a \textit{rho} \textsuperscript{0} tester strain (KL14–4A/60), indicating that the respiratory defect is caused by a nuclear recessive mutation and not by a deficiency of mitochondrial DNA.

The mutant SM45 is devoid of dithionite-reducible cytochrome \textit{b} while the amounts of cytochromes \textit{a} and \textit{a} \textsubscript{3} are not markedly modified, indicating a specific defect at the level of the cytochrome \textit{bc} \textsubscript{1} complex (Fig. 2). A complementing DNA fragment was selected by transformation of the mutant SM45 with a plasmid library of yeast nuclear DNA. The restriction enzyme digestion pattern of the fragment suggested the presence of the \textit{COR2} gene (17), and this was confirmed by complementation of a strain carrying a null allele in core protein 2

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2 E. Coissac, Gif-sur-Yvette, France, personal communication.
CCW30 is a mitochondrial cytoductant of CCB2 into CW30. CW30/SM45 is derived from CW30. CCB2 is a mitochondrial cytoductant of SM45/CB2 into JC8/55. CB10–2a was obtained by random sporulation of diploids issued from the cross between CW30/SM45 and KL14–4A/60. CHR2:40k is a mitochondrial cytoductant of CCB2 into HR2:40k. The strains CW30, CCW30, CW30/SM45, SM45/CB2, CCB2, and CHR2:40k contain the mitochondrial genome devoid of all introns (46).

As expected, no immunological response corresponding to core protein 2 was detected in the mutant carrying the cor2–45 mutation (Fig. 4, lane 2). We did not observe any signal that could correspond to the mutated protein. A possible explanation is that the antibodies react with the C-terminal half of core protein 2, which is lacking in the mutant, or that the mutated protein is more prone to proteolytic degradation.

As shown previously (18) (see also Figs. 1 and 2), a mutant carrying a null allele in the core protein 2 gene grows slowly on non-fermentable substrates and exhibits a low level of cytochrome c reductase activity. Surprisingly, the growth on non-fermentable substrates as well as the cytochrome c reductase activity are virtually abolished by the cor2–45 mutation (Figs. 1 and 2). Moreover, only traces of core protein 1 are present in cells carrying the cor2–45 mutation (Fig. 4, lane 2), whereas core protein 1 is at a nearly normal level in the core 2 deletion strain (Fig. 4, lane 5).

From these results, it is clear that the cor2–45 frameshift mutation has more severe effects than a deletion allele in the COR2 gene. As shown in Fig. 3, the cor2–45 mutation presumably leads to the synthesis of a truncated protein with a net charge of +15 versus –1 for the wild-type protein. This drastic charge and size modification could be responsible for the toxic effects of cor2–45, for instance, by allowing potentially harmful protein-protein interactions between the modified core protein 2 and other subunits of the cytochrome bc1 complex. This led us, as described in the next section, to search for extragenic suppressor mutations, in order to see if the effects of the cor2–45 mutation can be alleviated by a second mutation in another subunit of the complex.

A Mutation of the Mitochondrial DNA Replacing Serine 223 by Proline in Cytochrome b Restores Assembly and Activity of the Cytochrome bc1 Complex in the Nuclear Mutant Deficient for Core Protein 2—Only 1 revertant (SM45/CB2) out of 22 isolates was due to a mutation in mtDNA. The mutation was mapped by rho– deletion mapping to the mitochondrial cytochrome b gene (Table II) and sequenced. The suppressor mutation leads to replacement of the serine codon 223 (TCA) by a proline codon (CCA).

The revertant SM45/CB2 grows on glycerol medium (Fig. 1), exhibits ubiquinol-cytochrome c reductase activity of at least 60% of that of the wild type (Fig. 2, right panel), and spectrally detectable cytochrome b is partially restored (Fig. 2, left panel). In addition, core protein 1, which is dramatically decreased by the cor2–45 mutation, is present at a nearly normal level in the revertant (Fig. 4, lane 3).
The strains from top to bottom are CW30, wild type (COR2), HR2::40K0 and the mtDNA of the revertant SM45/CB2 (Table I). Analysis of this strain (CHR2::40K0) confirmed in the present work. The cor2–45 mutation consists of a single G deletion that modifies all of the codons after codon 165. As a result, the last 204 residues at the C terminus of the wild-type protein are replaced in the mutant by 32 novel residues.

As a control, we tested whether the S223P mutation in cytochrome b gives a phenotype when associated with the wild-type core protein 2. To this end, we constructed a strain having the nucleus of the wild-type strain CW30 and the mtDNA of the revertant SM45/CB2 (Table I). This strain (CCW30) exhibits normal cytochrome b content and grows normally on glycerol medium (Fig. 2), indicating that the S223P mutation in cytochrome b by itself has no deleterious effects on the cytochrome bc1 complex.

**DISCUSSION**

In the studies reported here, we first describe a frameshift mutation (cor2–45) in the nuclear COR2 gene by which the last 204 residues of core protein 2 are replaced by 32 novel residues and show that this mutation severely impairs assembly of the cytochrome bc1 complex. We then show that assembly of an active bc1 complex is restored by a single base pair transition in mitochondrial DNA, which leads to replacement of serine 223 by proline in cytochrome b. This is the first known example of a homoplastic missense point mutation of the mitochondrial DNA acting as a functional suppressor of a mutation located in a nuclear gene.

Replacement of serine 223 by proline in cytochrome b not only corrects the cytochrome bc1 complex deficiency resulting from the original frameshift mutation, but it also obviates the need for the core 2 protein since it alleviates the respiratory deficiency resulting from its complete deletion. It should be noted that the frameshift mutation strain is more responsive to...
the suppression by the cytochrome \( b \) mutation than is the cor2 deletion strain (60% and 41%, respectively, in terms of activity relative to the wild type are recovered by the S223P replacement). It is possible that the remaining part of core protein 2 in cor2–45 (about half of the wild-type protein) helps the assembly of or stabilize the bc1 complex, which could explain the observed differences in terms of activity and growth rates of the corresponding strains. These unexpected results illustrate unequivocally that the core 2 subunit of the mitochondrial cytochrome bc1 complex is not essential for the electron transfer

### TABLE II

**Location of the SM45/CB2 suppressor mutation in the cytochrome \( b \) gene by rho− deletion mapping**

The revertant strain SM45/CB2 was derived from the nuclear respiratory deficient mutant SM45 and shown to carry a suppressor mutation located in mtDNA. In order to localize the suppressor on mtDNA, rho− clones were derived by mild ethidium bromide treatment of the revertant strain and were tested for the retention or loss of the suppressor mutation by crossing to the original mutant. Forty rho− clones that retained the suppressor mutation were crossed pairwise with four mit− mutants previously located in mtDNA: cob-M7622 and cob-M4410 in the cytb gene, asxI-V45 in the coxI gene, and axsI-G481 in the coxl gene. +/− denotes the presence/absence of wild-type respiratory competent recombinants in the diploid progeny as indicated by growth on glycerol medium after two days at 28 °C. All the rho− tested rescued the cytb mutants, whereas only a minor fraction of them rescued the coxII and coxl mutants, showing that the suppressor is in cytb. The cob-M7622 mutation is at codon 33 of cytb (G33D, Ref. 42); cob-M4410 is at codon 221 (M221K, Ref. 43).

| coxl | cytb | coxI |
|------|------|------|
| asxI-V45 | cob-M7622 | cob-M4410 |
| + | + | − |
| − | + | + |

### FIG. 5.

The S223 residue of yeast mitochondrial cytochrome \( b \) belongs to a matrix-exposed hydrophilic domain. The figure represents the generally accepted eight-helix model of yeast cytochrome \( b \) (1, 33–38, 41–49). The black circled amino acids are modified in *S. cerevisiae* inhibitor-resistant mutants. DIU, diuron; ANA, antimycin; FUN, funiculosin; MYX, myxothiazol; MUC, mucidin; and STI, stigmatellin. As shown in this work, replacement of serine 223 by proline restores the assembly and activity of the cytochrome bc1 complex in a mutant deficient for core protein 2. According to the eight-helix model, the S223 residue belongs to an extra membrane hydrophilic domain facing the matrix phase.
dues (Fig. 3), could block the proper folding of core 1, thus making this subunit sensitive to proteolytic degradation. If the core proteins bind to cytochrome b, as suggested by the loss of the cytochrome in core protein-deficient yeast strains, the restoration of core protein 1 by the revertant could simply be due to a conformational change in cytochrome b that strengthens its interaction with core protein 1 and thus protects against any deleterious interaction with the mutated core protein 2.

More important, however, is that a single mutation, S223P, in one of its matrix-exposed connecting loops makes the cytochrome b resistant to mitochondrial protease, thus obviating the possibility of further proteolysis by a mitochondrial protease. The S223P mutation may render the matrix-exposed loop of the cytochrome intrinsically resistant to proteolytic activity, and thus protect it from proteolysis by a mitochondrial protease. The S223P mutation may render the matrix-exposed loop of the cytochrome b1 complex, the core proteins of the cytochrome b1 complex are proteolytically active (6, 7). In yeast, the core proteins located in the cytochrome b1 complex appear to be proteolytically inactive homologues of α-MPP and β-MPP (6, 12), which are localized in the matrix (14–16).

From this relationship and the peripheral location of the core proteins at the matrix side of the bc1 complex, we speculate that the proteolytically inactive core proteins bind to the matrix-exposed loop of cytochrome b and thus protect it from proteolysis by a mitochondrial protease. The S223P mutation may render the matrix-exposed loop of the cytochrome intrinsically resistant to mitochondrial protease, thus obviating the requirement for the core proteins. Notably, the cytochromes b in N. crassa and plant mitochondria, in which the core proteins have been shown to be proteolytically active, and likewise those of most eukaryotic mitochondria except yeast, contain a proline at the position equivalent to serine 223 of yeast (35). Furthermore, a single serine to proline mutation proximal to the MPP cleavage site in the presequence of the iron-sulfur protein of the yeast cytochrome bc1 complex blocks proteolysis by MPP.3

Given the complex evolutionary relationships between the core proteins and MPP and the genetic data presented in this study, it will be particularly interesting to see if, in yeast, the core proteins function to protect the cytochrome b against mitochondrial proteases. Further experiments to test this hypothesis are in progress.

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