**COQ9, a New Gene Required for the Biosynthesis of Coenzyme Q in Saccharomyces cerevisiae**

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Currently, eight genes are known to be involved in coenzyme Q biosynthesis in Saccharomyces cerevisiae. Here, we report a new gene designated COQ9 that is also required for the biosynthesis of this lipid quinone. The respiratory-deficient pet mutant C92 was found to be deficient in coenzyme Q and to have low mitochondrial NADH-cytochrome c reductase activity, which could be restored by addition of coenzyme Q. The mutant was used to clone COQ9, corresponding to reading frame YLR201c on chromosome XII. The respiratory defect of C92 is complemented by COQ9 and suppressed by COQ8/ABC1. The latter gene has been shown to be required for coenzyme Q biosynthesis in yeast and bacteria. Suppression by COQ8/ABC1 of C92, but not other coq9 mutants tested, has been related to an increase in the mitochondrial concentration of several enzymes of the pathway. Coq9p may either catalyze a reaction in the coenzyme Q biosynthetic pathway or have a regulatory role similar to that proposed for Coq8p.

Biosynthesis of coenzyme Q (ubiquinone) in eukaryotes occurs in mitochondria. Eight genes designated COQ1–8 have been shown to be involved in the biosynthesis of this lipid component of the electron transport chain of Saccharomyces cerevisiae (1). The products of these genes have been localized to the inner membrane (2–8) and, in some cases, were inferred to be present in a complex (4, 9, 10). The chemical intermediates detected in some mutants have revealed that the pathways in bacteria and in yeast are identical up to the formation of 3-hexaprenyl-4-hydroxybenzoic acid (HHB) (11, 12), at which point they diverge for the next three steps, but then converge again in the last stages of biosynthesis (11, 12).

A hallmark of most yeast coq mutants is the accumulation of HHB when the biochemical block occurs at any step subsequent to the formation of this early intermediate (5–8, 13, 14). This may indicate that 1) most intermediates of the pathway are unstable and degraded; 2) the pathway is highly regulated; or 3) the enzymes are organized in a complex, which is highly sensitive to mutations in any one of its components. The latter possibility is supported by recent evidence indicating an interdependence of some Coq proteins for their stability (4). Most of the COQ gene products have been related to specific reactions of the eukaryotic pathway based on their homology to the bacterial counterparts (6, 7). The reactions catalyzed by the products of COQ4 and COQ8/ABC1, however, still need to be clarified (5, 8).

In the course of analyzing the biochemical defects of respiratory-deficient pet mutants of *S. cerevisiae*, we have identified a new gene that, when mutated, produces a phenotype similar to that of coenzyme Q mutants. This gene is defined by complementation group G61 of our pet mutant collection (1) and has been named COQ9. Homologs of COQ9 are present in a wide range of different eukaryotes, but not in bacteria, indicating that its function is specific to coenzyme Q biosynthesis in mitochondria. Suppression of a coq9 mutant by COQ8/ABC1 (8, 16) suggests that the products of these two genes may be functionally related.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The strains of yeast used in this study are listed in Table I. The respiratory-deficient mutants of complementation group G61 were derived from *S. cerevisiae* strain D273-10B/A1 or D273-10B/A21 by mutagenesis with nitrosoguanidine or ethylmethane sulfonate (1). The following media were used routinely to grow yeast: 1% yeast extract, 2% glucose; 2% galactose; and 1% yeast extract, 2% ethanol, 2% peptone, and 3% glycerol.

**Cloning of COQ9 and COQ8**—Recombinant plasmids containing COQ9 (pG61/T2) and COQ8/ABC1 (pG61/T3) were isolated by transformation of C92/UL1 with a yeast genomic library consisting of partial Sau3A fragments of yeast nuclear DNA cloned in the yeast/Escherichia coli shuttle plasmid YEp24 (18). Approximately 5 × 10⁶ cells were transformed with 50 μg of the library DNA by the method of Schiestl and Gietz (19).

**Disruption of COQ9**—The linear KpnI fragment containing COQ9 was transferred to YEp352K. This plasmid is identical to YEp352 (20) except that it has only the KpnI site of the multiple cloning sequence. This construct was linearized at the SacI site inside the COQ9 coding sequence and ligated to a 1-kb SacI fragment containing the yeast URA3 gene. The linear KpnI fragment with the disrupted allele was substituted for the wild-type gene in strain W303 by the one-step gene replacement procedure (21).

**Construction of Hybrid Genes Expressing Coq9p Tagged with Hemagglutinin (HA) and Coq8p Tagged with Glutathione S-Transferase (GST)**—At Their C Termini, Respectively—The COQ9 coding sequence plus 359 nucleotides of the 5′-untranslated region was amplified with primers 5′-ggaattcggagggagccggtcatttc-3′ and 5′-ggaaagccttcaagctttggtggccgac-3′ and ligated to a multiple cloning site containing the yeast URA3 gene. The linear KpnI fragment with the disrupted allele was substituted for the wild-type gene in strain W303 by the one-step gene replacement procedure (21).

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Lipid extracts were prepared from 1 mg of mitochondrial protein. The chromosomal DNA of a W303 COQ8. The resultant strain was grown in 600 ml of minimal medium (2% galactose and 0.1% dextrose) containing 6.34 μCi of 4-L-[1-14C]hydroxybenzoic acid (450 mCi/mmol; American Radiolabeled Chemicals, Inc. St. Louis, MO). Lipid extracts of cell pellets were prepared as described (13), dried completely under N2 gas, and stored at −20 °C. The dried lipids were resuspended in 50 μl of 100% acetonitrile and vortexed vigorously, and the tubes were centrifuged briefly to remove a small amount of a white precipitate. The clear supernatant (25 μl) was injected into an HPLC system as described (13) with the following modifications. A reverse-phase BetaBasic column (2.1 × 100 mm, Thermo Electron Corp.) was developed with a gradient of solvent A (50:50 acetonitrile/H2O and 0.01% trifluoroacetic acid) and solvent B (99:1 acetonitrile/H2O and 0.01% trifluoroacetic acid) at a flow rate of 400 μl/min. At the time of injection, solvent A/B was 50:50; and at 2 min, the percentage of solvent B was altered linearly so that solvent B was 100% at 9 min and remained at 100% until 25 min. The retention time for coenzyme Q6 measured at 272 nm was 22 min, with a delay time of 0.37 min due to the fraction collector. Fractions were collected every minute, and the radioactivity was measured by scintillation counting in 10 ml of ScintiVerse (Fisher).

**RESULTS**

**Phenotype of coq9 Mutants**—C92 is one of seven independent isolates assigned to complementation group G61 of a pet mutant collection (1). The mutants grow on glucose, but not ethanol and/or glycerol, as a carbon source, indicating a defect in respiration. The respiratory deficiency of G61 mutants stems from recessive mutations in a nuclear gene, as they are complemented by a pα strain with a normal complement of nuclear genes but lacking mitochondrial DNA.

The spectra of mitochondria from C92 indicated reduced reductase and cytochrome activities of the mitochondria, both of which were reduced in the mutants (Table II). This is also evident from the NADH-cytochrome reductase activity in the mutant was <10% of that measured in the wild-type strain, even though the presence of cytochrome b in the spectrum of the mutant mitochondria indicated a
substantial amount of the cytochrome bc1 complex. This phenotype could be explained by a defect in NADH dehydrogenase, the cytochrome bc1 complex, or coenzyme Q6, all of which participate in the reduction of cytochrome c. The restoration of NADH-cytochrome c reductase by addition of exogenous coenzyme Q6 (Table II) excluded a defect in either NADH dehydrogenase or the cytochrome bc1 complex; rather, it pointed to a deficiency in coenzyme Q as the explanation for the respiratory defect.

This was further tested by analyzing the coenzyme Q6 content of mitochondria from the wild-type and mutant strains. Lipid extracts of mitochondria were separated by reverse-phase chromatography on a C18 column. On this column, coenzyme Q6 was detected in similar extracts from the wild-type strain and the coq9 null mutant with a 4-[U-14C]hydroxybenzoic acid. The wild-type strain incorporated the precursor mainly into coenzyme Q66, although some of the label was also present in a polar material eluting at 16 min. This radiolabeled compound was presumed to be HHB based on our previous characterization of lipid extracts from cells labeled under these conditions (13). In that study, HHB was identified by purification, chemical derivation, and confirmation of its structure by mass spectrometry analyses. HHB is an early intermediate in coenzyme Q biosynthesis and has been shown to be a predominant intermediate in wild-type yeast (13). In contrast, there was no detectable radioactivity at the position of coenzyme Q6 in the coq9 mutant, and the elution of radiolabeled material at 16 min is consistent with the presence of HHB, indicating that the lesion occurs after the prenylation step (Fig. 2B).

Cloning of COQ9—C92/UL1, a derivative of C92 with the leu2 and ura3 markers, was transformed with a yeast genomic plasmid library. Restriction analysis of the nuclear DNA inserts in plasmids isolated from respiratory-competent transformants indicated three distinct and non-overlapping regions of DNA. The genes responsible for conferring respiration in C92/UL1 were identified by testing the ability of different regions of the cloned nuclear DNA inserts to restore the ability of the mutant to grow on glycerol. The results of the subcloning indicated that rescue of the mutant by the plasmid designated pG61/T2 depended on the unknown reading frame YLR201c (Fig. 3), whereas in pG61/T3, the gene responsible for conferring respiration is COQ8/ABC1 (8, 16). Complementation tests with subclones from the third plasmid, pG61/T4, identified the multicopy suppressor as DTD1 (data not shown), the gene for D-Tyr-tRNA[Tyr] deacylase (27), which was previously shown to counter the suppressor activity of sup45-2 in yeast (28). The mechanism by which DTD1 rescues the coq9 mutant has not been further studied. Two other mutants from complementation group G61 (E40 and E270) were also tested and found to be complemented by YLR201c. However, the respiratory deficiencies of E40 and E270 were not rescued by either COQ8/ABC1 or pG61/T4 (Fig. 4). The ability of C92/UL1 to grow normally on non-fermentable substrates when transformed with YLR201c and the lack of complementation of C92/UL1 by W303ΔCOQ9, a mutant carrying a deletion of YLR201c, strongly suggest that the restoration of respiration by this gene is due to complementation. In contrast to YLR201c, COQ8/ABC1 was ascertained to be a suppressor based on its specificity for only one of the three mutant alleles tested and complementation of C92 and W303ΔCOQ9 by a coq8abc1 mutant. These observations imply that the growth defect of C92 and other G61 mutants stems from mutations in reading frame YLR201c. This reading frame has been named COQ9 in keeping with the earlier convention for naming genes involved in coenzyme Q biosynthesis in yeast (1).

Disruption of COQ9—COQ9 was disrupted with the yeast URA3 gene at the internal SacI site. The resultant strain was respiratory-deficient (not shown), and the spectrum of mitochondria disclosed a partial pleiotropy similar to that of C92, resulting in some reduction of cytochromes a and b (Fig. 1). Assays of respiration in the null mutant confirmed that the NADH-cytochrome c reductase and succinate oxidase activities of the mutant mitochondria were reconstituted by coq9 (Fig. 5 and Table II).

Localization and Sizing of Coq9p—To localize its product, COQ9 was fused in-frame at its 3′-end to a sequence coding for nine amino acids constituting the HA tag. The fusion gene was able to fully complement the null mutant W303ΔCOQ9, even when it was integrated in a single copy into nuclear DNA. Like other enzymes of the coenzyme Q biosynthetic pathway in yeast (4–8), Coq9p was detected in mitochondria, but not in the

**TABLE II**

| Strain     | NADH-cytochrome c reductase | Cytochrome oxidase activities of mitochondria |
|------------|-----------------------------|---------------------------------------------|
|            | − Coenzyme Q6              | + Coenzyme Q6 |
|            | μmol cytochrome c oxidized or reduced per min/protein | |
| W303-1A    | 2.15 ± 0.08                | 2.21 ± 0.07 |
| C92        | 0.21 ± 0.02                | 1.90 ± 0.05 |
| aW303ΔCOQ9 | 0.14 ± 0.01                | 1.67 ± 0.02 |

FIG. 1. Spectra of mitochondrial cytochromes in the wild-type strain and G61 mutants. Mitochondria were prepared from the respiratory-competent parental wild-type (WT) strain W303-1B, C92, and W303ΔCOQ9 (a respiratory-deficient mutant with a disrupted copy of COQ9). The mitochondria were extracted at a protein concentration of 5 mg/ml in the presence of 1% potassium deoxycholate and 1 mM KCl (25). The spectra of the extracts were obtained at room temperature following oxidation of the sample in the reference cuvette with potassium ferricyanide and reduction of the sample cuvette with sodium dithionite. The absorption bands corresponding to cytochromes c, c1, b, a, and a3 are indicated.
The intramitochondrial distribution of the HA-tagged protein (Coq9p-HA) was studied by testing its sensitivity to proteinase K in mitochondria and in mitoplasts lacking an intact outer membrane. The results of Fig. 6B indicate that the hypotonic treatment effectively converted mitochondria to mitoplasts because the latter were almost completely devoid of cytochrome b$_2$, a soluble marker for...
the intermembrane space. This was also supported by the susceptibility to proteinase K of Sco1p, an inner membrane protein facing the intermembrane space (33). The retention of susceptibility to proteinase K of Sco1p, an inner membrane
protein (data not shown). Genetic evidence that some enzymes of coenzyme Q biosynthesis exist in a complex (4) made it of interest to determine the native size of Coq9p. Coq9p-HA was extracted from mitochondria of W303ΔCOQ9/ST8 with dodecyl maltoside, and the extract was centrifuged through a linear sucrose gradient (Fig. 7A). The HA-tagged protein sedimented midway between lactate dehydrogenase (140 kDa) and hemoglobin (64 kDa), suggesting a mass in the range of 100 kDa. The sedimentation characteristics of several other enzymes of coenzyme Q biosynthesis were also examined. Both Coq3p and Coq5p sedimented similarly to Coq9p-HA, whereas Coq4p was more broadly distributed in fractions spanning the higher molecular mass region of the gradient (Fig. 7A).

Steady-state Levels of Coenzyme Q Biosynthetic Enzymes in the coq9 Mutant—The absence of some enzymes of coenzyme Q

FIG. 5. NADH and succinate oxidase activities of mitochondria from the wild-type strain and a coq9 null mutant. Mitochondria from the wild-type (WT) strain W303-1B and from the coq9 null mutant W303ΔCOQ9 were used to measure succinate and NADH oxidase activities at 25 °C in 0.86 ml of solution containing 10 mM potassium phosphate buffer (pH 7.5) as described previously (29). Additions of 0.09 mg of wild-type or 0.3 mg of mutant mitochondria (mit), 1 μmol of NADH, 1 μmol of succinate (succ.), 20 nmol of coenzyme Q1 (CoQ1), and 1 μmol of KCN are indicated. The specific activities (μmol of O2/min/mg of protein) are shown in the insets of each panel.

FIG. 6. Localization of Coq9p. A, mitochondria (Mit) and the post-mitochondrial supernatant fraction (PMS) consisting mostly of soluble cytosolic proteins were isolated from the coq9 null mutant expressing Coq9p-HA either from a multicopy plasmid (ST7) or from a chromosomally integrated copy of the hybrid gene (ST8). Mitochondrial (20 μg) and post-mitochondrial supernatant proteins (40 μg) were separated by SDS-PAGE (30) on a 12% polyacrylamide gel and transferred to nitrocellulose. The Western blot was reacted with a mouse monoclonal antibody against the HA tag. Proteins were visualized using the SuperSignal chemiluminescent substrate kit (Pierce) following a secondary reaction with peroxidase-conjugated anti-mouse IgG (Sigma). B, mitochondria were prepared from the coq9 null mutant with the chromosomally integrated fusion gene expressing Coq9p-HA (W303ΔCOQ9/ST8) by the method of Glick and Pon (31). Mitochondria (Mit) were converted to mitoplasts (Mp) by the method of Glick (32). Both mitochondria and mitoplasts were incubated on ice for 1 h in the absence or presence of 0.1 mg/ml proteinase K (Prot K). Following addition of phenylmethylsulfonyl fluoride to inhibit proteinase K, mitochondria were isolated by centrifugation for 10 min at 14,000 rpm. Equivalent amounts (40 μg) of mitochondria and mitoplasts were separated by SDS-PAGE and further processed as described for A with antisera against HA, Sco1p, cytochrome b2 (Cyt. b2), and α-ketoglutarate dehydrogenase (α-KGD). C, W303ΔCOQ9/ST8 mitochondria were sonically irradiated for 5 s with a Branson sonifier and centrifuged at 300,000 × g, for 15 min. The supernatant (Sup) consisting of soluble proteins from the matrix and intermembrane space was collected, and the pellet consisting of submitochondrial particles (SMP) was suspended in the starting volume of buffer. Equivalent volumes of mitochondria (40 μg), supernatant, and submitochondrial particles were separated by SDS-PAGE and treated either with a monoclonal antibody against the HA tag or with a polyclonal antibody against subunit 4 of cytochrome oxidase (Cox4p).
biosynthesis has been shown to cause reductions in the concentrations of one or more of the other enzymes of this mitochondrial pathway in yeast (4). For example, null mutations in any one of the eight COQ genes elicit a marked reduction in the steady-state levels of Coq4p and Coq6p, but not Coq5p. To determine whether this is also true of the coq9 mutant, total mitochondrial proteins from the wild-type strain and from several coq9 null mutants were separated by SDS-PAGE and, after transfer to nitrocellulose, were probed with antibodies against Coq3p, Coq4p, and Coq5p. The antibody-antigen complexes were visualized using the SuperSignal detection system after a second reaction with peroxidase-conjugated anti-mouse or anti-rabbit IgG. The gradient fractions were also analyzed for the distribution of hemoglobin (Aα111) and lactate dehydrogenase (LDH) by measuring the pyruvate-dependent oxidation of NADH at 340 nm. B, mitochondria from W303-1A (wild-type strain), W303Δcoq9 (coq9 null mutant), C92/UL3 (coq9 point mutant), C92/UL3/T3 (coq9 point mutant transformed with the COQ8/ABC1 gene on a high copy plasmid), and W303Δcoq5 (coq5 null mutant) were separated by SDS-PAGE and further processed as described for A.

Coq9p and Coq8p Are Not Stably Complexed to Each Other—
The genetic interaction of COQ8 and COQ9 as evidenced by the allele-specific suppression of a coq9 mutant by COQ8 suggested that the products of these two genes might also interact physically. For technical reasons, attempts to answer this question by co-immunoprecipitation or tag-dependent pull-down assays did not succeed. In view of this, we examined the sedimentation properties of the two proteins in different genetic backgrounds. The strain used was W303Δcoq8Δ9/st3/st8, a double mutant in which the deleted COQ8 and COQ9 genes were replaced with chromosomally integrated genes expressing Coq8p-GST and Coq9p-HA. The tagged genes conferred wild-type growth of the double mutant on glycerol/ethanol and expressed both proteins (data not shown). The sedimentation of each protein was also examined in double mutants expressing each Coq8p-GST (W303Δcoq8Δ9/st3) or Coq9p-HA (W303Δcoq8Δ9/st8) alone. The results of these experiments failed to disclose the presence of a stable complex of the two proteins. The sedimentation of Coq8p-GST and Coq9p-HA was different in the strain expressing both (Fig. 8A). Furthermore, the sedimentation of each protein with respect to the molecular mass markers was not visibly different in the strains expressing only one of the two proteins (Fig. 8, B and C).

**DISCUSSION**

*S. cerevisiae* and probably eukaryotic organisms in general convert 4-hydroxybenzoic acid to coenzyme Q in mitochondria (11, 34–36). Although the basic chemistry of coenzyme Q biosynthesis is known and many of the gene products have been characterized, this does not by any means exclude the existence...
of other genes coding for still unidentified components of the pathway. Additionally, very little is currently known about the regulation of coenzyme Q biosynthesis. These circumstances warrant further screens of pet mutants for lesions in gene products required for the biosynthesis and regulation of this important electron carrier.

Here, we present evidence that reading frame YLR201c on chromosome XII of the S. cerevisiae genome, which we have named COQ9, codes for a mitochondrial inner membrane protein that functions in coenzyme Q biosynthesis. This is supported by the absence of coenzyme Q6 in coq9 null point mutants and restoration of coenzyme Q-dependent respiration of mutant mitochondria by supplementation of the water-soluble analog coenzyme Q2.

A recent study has revealed that the steady-state concentrations of some enzymes of the coenzyme Q6 biosynthetic pathway are dramatically reduced in mutants harboring mutations in many of the COQ genes. A similar decrease in the methytransferases encoded by COQ3 (2) and COQ5 (6) is seen in the coq9 null mutant, which is also partially deficient in Coq4p. The function of the COQ4 product in coenzyme Q6 biosynthesis remains to be elucidated (5). These pleiotropic effects have been interpreted to indicate that some of the enzymes of the pathway exist in a complex that is destabilized either by the absence of one of its constituents or by the failure of the mutant to synthesize coenzyme Q6 or an early intermediate of coenzyme Q6 (4). In turn, this might cause an increased turnover of other constituents of the complex, particularly proteins that are prone to proteolytic degradation when no longer protected by their partners. The existence of such a complex(s) is supported by more recent direct evidence (10). The decrease in the steady-state concentrations of Coq3p, Coq4p, and Coq5p in the coq9 mutant is therefore consistent with a role of Coq9p in coenzyme Q biosynthesis.

The mass of Coq9p deduced from the gene sequence is 29.9 kDa. The N-terminal 15–20 residues probably correspond to a COQ8 ABC1 or is complexed to other proteins. Coq3p and Coq5p, which co-sediment with Coq9p in sucrose gradients, are candidates to be part of such a complex.

An important finding is the ability of COQ8/ABC1 to suppress the coenzyme Q deficit of the coq9 point mutant C92. COQ8/ABC1 does not suppress the coq9 null mutant or two other coq9 mutants from complementation group G61. Overexpression of COQ8 does not rescue other coq mutants. Suppression of the coq9 point mutant by COQ8 correlates with a partial restoration of coenzyme Q6 in mitochondria. Like mutations in other COQ genes, those in COQ8/ABC1 express a deficiency in NADH- and succinate-cytochrome c reductases (25). Studies of coq8/abc1 mutants showed that their primary lesion is in coenzyme Q biosynthesis (8). COQ8/ABC1 was also identified in a previous study as a high copy suppressor of a cbs2-223 mutant (16) blocked in translation of cytochrome b (37). Subsequent studies indicated, however, that the suppression is specific to the premature UGA termination codon in the cbs2-223 mutant and is mediated not by ABC1, but by the downstream tryptophanyl-tRNA_{ca} by a well documented mechanism (38, 39).

Although Coq8p has not been demonstrated to have protein kinase activity, its sequence contains motifs present in members of a large eukaryotic protein kinase superfamily (40). Mutations in ubiB and aarF, the E. coli and Providencia Stu-artii homologs of COQ8/ABC1, result in accumulation of poly-preneylphenol (41), the precursor for the first hydroxylation step in the bacterial pathway (15). Because Coq8p/Abc1p is not homologous to any known monooxygenases, a plausible hypothesis is that it regulates either hydroxylation or some other step in the pathway by direct phosphorylation of the responsible enzyme or through a signaling pathway that activates coenzyme Q biosynthesis. Coq9p is also not homologous to either monooxygenases or methylases.

The allele specificity of suppression of coq9 mutants by COQ8 points to a genetic and possibly physical interaction of the proteins encoded by these two genes. However, such an interaction cannot be a stable one based on the different sedimentation properties of the two proteins and the lack of influence on the sedimentation of either protein by the absence of the other. Despite our failure to detect a stable complex of Coq8p and Coq9p, a transient interaction of the two cannot be excluded. Independent of whether the two proteins interact physically, the ability of COQ8 to suppress a coq9 mutant suggests that Coq9p may be a regulatory protein. Alternatively, Coq9p could catalyze a reaction in the pathway proper. In this case, suppression of the C92 allele by overexpression of COQ8 may result from a combined up-regulation of the other enzymes of the pathway and the retention by the Coq9p mutant in C92 of some residual enzyme activity.

Like other coenzyme Q-deficient strains (5–8, 13, 14), the coq9 mutants reported here are able to incorporate 4-hydroxybenzoic acid into HHH. This indicates that the COQ9 product acts subsequent to the prenylation of 4-hydroxybenzoic acid.

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