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Yeast Clk-1 Homologue (Coq7/Cat5) Is a Mitochondrial Protein in Coenzyme Q Synthesis*

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Mutations in the *clk-1* gene result in slower development and increased life span in *Caenorhabditis elegans*. The *Saccharomyces cerevisiae* homologue *COQ7/CAT5* is essential for several metabolic pathways including ubiquinone biosynthesis, respiration, and gluconeogenic gene activation. We show here that Coq7p/Cat5p is a mitochondrial inner membrane protein directly involved in ubiquinone biosynthesis, and that the defect in gluconeogenic gene activation in *coq7/cat5* null mutants is a general consequence of a defect in respiration. These results obtained in the yeast model suggest that the effects on development and life span in *C. elegans clk-1* mutants may relate to changes in the amount of ubiquinone, an essential electron transport component and a lipid soluble antioxidant.

Research into the components responsible for controlling longevity have uncovered both environmental effects and genetic determinants (1, 2). The nematode *Caenorhabditis elegans* has been used as a model for many such studies. Multiple life-extension mutants have been identified which affect various aspects of development (3). One of the genes identified in determination of life span was identified as *Clock-1* (*clk-1*). *clk-1* mutants exhibit a pleiotropic phenotype, characterized by delayed embryonic and postembryonic development, a slowing of adult behaviors such as swimming, pharyngeal pumping, and defecation, and an extended life span (4). The *clk-1* mutants also have an increased resistance to stress induced by UV treatment (5). Recently the *C. elegans clk-1* gene was characterized and found to be conserved among eukaryotes, including humans, rodents, and the yeast *Saccharomyces cerevisiae* (6).

The yeast *clk-1* homologue was independently isolated as *COQ7* and *CAT5* (7, 8). The *COQ7* gene is required for the synthesis of ubiquinone (coenzyme Q or Q),[1] an isoprenylated benzoquinone that functions in the respiratory electron transport chain in the inner mitochondrial membrane of eukaryotes (9). Like other yeast *coq* mutants (10), the *coq7/cat5* mutants lack Q, are respiration defective, and are incapable of growing on nonfermentable carbon sources (8, 10). A yeast mutant harboring the *coq7-1* allele (encoding the substitution of Asp for Gly^{104}) was found to accumulate both 3-hexaprenyl-4-hydroxybenzoate (HHB) and a small amount of 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, two intermediates in Q biosynthesis (8). However, mutants with deletions in the *COQ7* gene produce only HHB. HHB is the predominant Q intermediate that accumulates in yeast mutants with deletions in any one of six *COQ* genes (*COQ3-COQ8*) (11). Transformation of either the *coq7-1* point mutant or the *coq7* null mutant with the yeast *COQ7* gene restored both growth on nonfermentable carbon sources and the synthesis of Q. These results led to the development of two models for Coq7p function in Q biosynthesis: (i) Coq7p may itself act in one or more monooxygenase steps in the pathway, and (ii) Coq7p provides a component of a multisubunit complex that is required for the conversion of HHB to Q (8, 11). Since the amino acid sequence shares no similarity to any known monooxygenase or hydroxylase proteins, there is little support for the first model. The *COQ7/CAT5* homologue from either rat or *C. elegans* rescued the yeast *coq7/cat5* mutant for growth on nonfermentable carbon sources, suggesting a conservation of function from yeast to animals (6, 12).

The yeast *COQ7* gene was independently isolated as *CAT5*, a gene required for the release of gluconeogenic genes from glucose repression (7). Glucose repression is a global regulatory system in *S. cerevisiae* that affects the transcription of genes involved in gluconeogenesis, alternative sugar metabolism, and respiration (13–15). Upon deletion of *CAT5*, binding of gene activators to the upstream activating sequences within gluconeogenic promoters was abolished resulting in a complete loss of gluconeogenic gene activation (7). These data provided support for a role of Cat5p in the cascade regulating gluconeogenic gene activation. Other genes necessary for the release

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[1] The abbreviations used are: Q, ubiquinone or coenzyme Q; QH_2, reduced ubiquinone or ubiquinol; HHB, 3-hexaprenyl-4-hydroxybenzoate; Coq7p, the polypeptide encoded by *COQ7*; Coq7–1p, the polypeptide encoded by the point mutant allele of the yeast *COQ7* gene; Cat5p, the polypeptide encoded by *CAT5*; Q_6, ubiquinone containing six isoprene units; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

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TABLE I
Genotypes and sources of S. cerevisiae strains

Strain	Genotype	Source or Ref.
CEN.PK2–1C	**a**, *his3-Δ1, leu2–3,112, trp1–289, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	P. Koetter, unpublished results.
CEN.PK130–7B	α, *his3-Δ1, leu2–3,112, trp1–289, ura3–52, cat1::HIS3, MAL2–8ᶜ, MAL3, SUC3*	P. Koetter, unpublished results.
CEN.PK131–8B	CEN.PK2–1C-*cat3::LEU2*	P. Koetter, unpublished results.
CEN.MP3–1A	CEN.PK2–1C-*cat5::HIS3*	7
CEN.NB1–1A	CEN.PK2–1C-*cat8::LEU2*	N. Bojunga, unpublished results.
EG103	α *his3Δ1, leu2–3,112, trp1–289, ura3–52*	84
FW103	EG103-*coq3::LEU2*	85
DO103	EG103-*atp2::LEU2*	82
WAY.5–4A	**a**, *his3-Δ1, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	86
ENY.MP7–3C	α, *cox7–7, leu2–3, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	86
ENY.MP30–4D	α, *mtf2–30, his3-Δ1, leu2–3, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	86
ENY.MP44–3A	α, *cox15–44, his3-Δ1, leu2–3, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	86
ENY.MP60–1C	α, *cox10–60, his3-Δ1, leu2–3, ura3–52, MAL2–8ᶜ, MAL3, SUC3*	86
JM43ΔCOQ7	α, *his4–580, leu2–3, 112, trp1–289, ura3–52, coq7Δ-1::LEU2*	8
W303.1B	α, *ade2–1, his3–11,15, leu2–3,112, trp1–1, ura3–1*	87
CC303	W303.1B-*coq3::LEU2*	82
W303ΔCOQ7	W303.1B-*coq7::LEU2*	8
CC304	W303.1B-*atp2::LEU2*	82
W303ΔCOR1	W303.1B-*cor1::HIS3*	40

from glucose repression were identified by the characterization of glucose derepression mutants *cat1* (*snf1*) (16, 17), *cat3* (*snf4*) (18, 19), and *cat8* (20). Expression of gluconeogenic genes requires the pleiotropic Cat1p·Cat3p protein kinase complex (21, 22) and the zinc cluster-transcriptional activator Cat8p (7, 20, 23). Since strains with mutations in genes mediating glucose repression (*cat1*, *cat3*, or *cat8*) were defective in activation of a *CAT5-lacZ* reporter gene, a coregulation of respiratory chain elements and gluconeogenesis was postulated (7).

Elucidation of the function of Coq7/Cat5p in yeast should provide insight regarding the function of *clk-1* in aging and development in *C. elegans*. The apparent dual function of Coq7p/Cat5p in yeast Q biosynthesis and glucose derepression raised the question of whether the observed defect in Q biosynthesis resulted from a defect in glucose derepression, or vice versa. In the present study the relationship between these functions is further investigated.

EXPERIMENTAL PROCEDURES

Strains and Growth Media—The strains of *S. cerevisiae* used in this study are described in Table I. Strains were grown in standard media as described (24). Growth and *in vivo* labeling of Q_6 with *p*-[U-^{14}C]hydroxybenzoic acid (365 Ci/mol) was as described (25). For derepression experiments cells were grown in glucose-containing medium to mid-log phase and then transferred to the respective ethanol containing medium for 6 h.

Rescue of coq Mutants with Exogenous Q_6—Yeast strains W303.1B (wild-type), W303ΔCOQ7 (*coq7Δ/cat5Δ*), CC303 (*coq3Δ*), CC304 (*atp2Δ*), and W303ΔCOR1 (*cor1Δ*) were grown overnight in 15 ml of YPD to stationary phase and then diluted into 40 ml of YPE ($OD_{600\ nm}$ = 0.6) with or without Q_6 supplementation (Sigma). Growth was monitored by $OD_{600\ nm}$ measurements, and at the same time samples were taken for enzymatic assays.

Enzyme Assays—Yeast crude extracts were prepared with glass beads (26) in 0.1 M potassium phosphate buffer and the protein concentration was determined using the bicinchoninic acid protein assay method (Pierce). Phosphoenolpyruvate carboxykinase and isocitrate lyase activities were measured as described (27, 28).

Plasmid Constructions—Two yeast expression plasmids, one single copy and one multiple copy, were constructed to express the Coq7 polypeptide containing a carboxyl-terminal peptide (MYPYDVPDYASLDGPMST) corresponding to the carboxyl terminus of the influenza hemagglutinin (HA) viral protein, an epitope for the 12CA5 monoclonal antibody (29). Construction was begun by directional cloning using *Sal*I and *Not*I sites in the plasmid pADCL (30). The *COQ7* yeast nucleotide sequence corresponding to the open reading frame was amplified by polymerase chain reaction with oligonucleotides containing *Sal*I and *Not*I linkers to allow for the in-frame ligation of the *COQ7* sequence. The sequences encoding the ADH promoter, *COQ7* sequence, HA-epitope, and termination site were then liberated from pADCL by *Bam*HI partial restriction enzyme digestion and subsequently ligated into the *Bam*HI site of two plasmids pRS316 and pRS426 (31) to give psHA71 and pmHA71 providing single and multiple copy plasmid maintenance in yeast, respectively. pNMQ71 is maintained in single copy and contains the *COQ7* nucleotide sequence and 414 base pairs of upstream sequence, constructed as described previously (8). Yeast cells were transformed with pNMQ71, psHA71, pmHA71, or pRS316 (32). Transformants were selected for the presence of the *URA3* gene on SD-Ura plates. The Ura^+ colonies were subsequently replica plated to YPG plate media. The Coq7-HA epitope fusion protein retains activity as assayed by the ability of either the single or multicopy plasmid construct to rescue *coq7/cat5* null mutant yeast strains for growth on media containing a nonfermentable carbon source (YPG plates, data not shown). One of the *coq7* null mutants used in the complementation above, JM43ΔCOQ7 (8), was used for subcellular localization by Western analysis.

Cell Lysis and Fractionation—Cell cultures (1 liter) were grown in semisynthetic lactate media to saturation density. Spheroplasts were prepared and lysed by Dounce homogenization with a tight fitting pestle as described (33) with one exception: protease inhibitors were prepared in dimethyl sulfoxide and added prior to cell lysis. Final concentrations of the protease inhibitors were as follows: benzamidine 1 mM, leupeptin 1 μg/ml, pepstatin 2 μg/ml, chymostatin 1 μg/ml, aprotinin 1 μg/ml, antipain 1 μg/ml. Purified mitochondria were isolated from a linear Nycodenz gradient as described (33).

Subfractionation of Purified Mitochondria—The outer mitochondrial membrane was broken by adding 5 volumes of ice-cold 20 mM HEPES-KOH, pH 7.4, to 2 mg of purified mitochondria. After a 10-min incubation on ice, the mixture was centrifuged in a microcentrifuge at 4 °C for 10 min. The supernatant contained the intermembrane space components while the pellet consisted of mitoplasts and disrupted outer membrane. The pellet was resuspended in 1 ml of 20 mM HEPES-KOH, pH 7.4. Integral and peripheral membrane proteins were separated via two methods: 1) alkaline carbonate extraction (34) or 2) extraction with the same conditions as the first method, but with 2 M urea in place of alkaline carbonate as the extracting agent (35).

Western Analysis—Fractions were assayed for protein concentration by the bicinchoninic acid assay (Pierce). Equal amounts of protein from the mitochondrial fractions of cells containing the plasmids pNMQ71, psHA71, and pmHA71 were analyzed by electrophoresis on 12% Tris glycine gels and subsequently transferred to Hybond ECL Nitrocellulose (Amersham). Western analysis and membrane stripping were performed as described by Amersham. An exception to the stated protocol was the use of Western washing buffer: 10 mM Tris, pH 8.0, 154 mM NaCl, 0.1% Triton X-100.

Polyclonal rabbit antisera were generated to detect Coq7p/Cat5p in a wild-type strain CEN.PK2–1C, and in both crude and purified mitochondria fractions. The *COQ7/CAT5* reading frame was amplified by polymerase chain reaction and inserted into pGEX-CS1 (Pharmacia, Piscataway, NJ) allowing for the isopropyl-1-thio-β-D-galactopyranoside inducible production of a 55-kDa GST-Coq7 fusion protein in *E. coli* RR1. The fusion protein was purified using preparative SDS-PAGE. Polyclonal rabbit antiserum against Coq7p/Cat5p was obtained by the standard immunization protocol (Eurogentec, Seraing, Belgium) with 1 mg of purified fusion protein. The primary antibodies were used at the

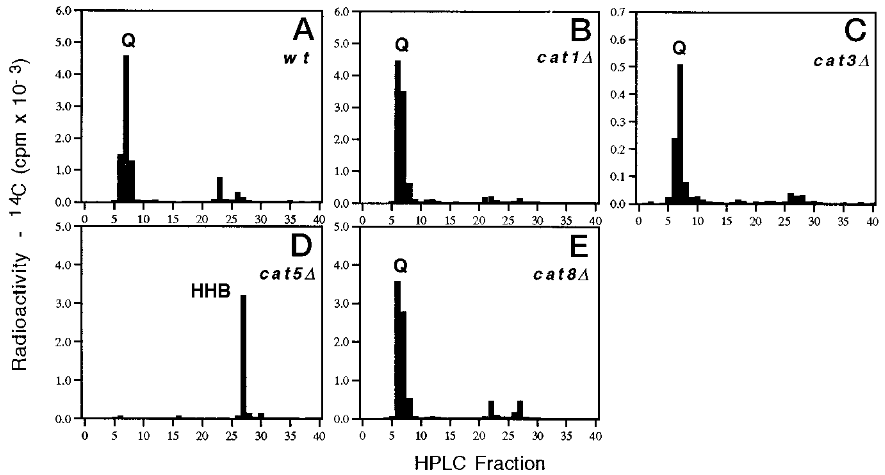

FIG. 1. **Yeast strains with mutations in *CAT1, CAT3,* or *CAT8* synthesize Q.** Yeast strains were grown in the presence of *p*-[U-^{14}C]hydroxybenzoic acid as described under "Experimental Procedures." Lipid extracts were separated by normal phase high performance liquid chromatography (CN column, Zorbax, 4.6 × 250 mm, Mac Mod Analytical, Chadds Ford, PA), and determination of radioactivity in each 1-ml fraction were as described (25). Strains are indicated: CEN.PK2–1C (wild-type), CEN.PK130–7B (*cat1*Δ), CEN. PK131–8B (*cat3*Δ), CEN.MP3–1A (*coq7*Δ/*cat5*Δ), and CEN.NB1–1A (*cat8*Δ).

following dilutions: 12CA5, 1:10,000; OM45, 1:1,000; Mas2, 1:1,000; Sec62p, 1:500; Kex2p, 1:800; ALP, 1:3,000; Hsp60, 1:10,000; $F_1\beta$-ATPase, 1:10,000; cyt c_1, 1:200; GST-Coq7p polyclonal antiserum, 1:5,000. Horseradish peroxidase-linked secondary antibodies to rabbit and mouse IgG (Amersham) were used in a 1:1,000 dilution, and alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) was used in a 1:5,000 dilution.

RESULTS

Defects in Glucose Derepression Do Not Impair Q Biosynthesis—To assay whether Q biosynthesis is affected by mutations in the glucose derepression system, yeast strains harboring mutations in *cat1*, *cat3*, or *cat8* were tested for their ability to synthesize Q. A total lipid extract prepared from wild-type yeast grown in the presence of *p*-[U-^{14}C]hydroxybenzoic acid and separated by normal phase high performance liquid chromatography gives rise to a peak of radiolabeled material that co-elutes with a Q_6 standard (fractions 6 and 7, Fig. 1*A*). When this same procedure is performed on radiolabeled lipid extracts from the *cat1*, *cat3*, or *cat8* mutants, production of Q_6 is still observed (Fig. 1, *B, C*, and *E*). Thus neither the pleiotropic Cat1p/Cat3p (Snf1p/Snf4p) protein kinase involved in glucose derepression nor the transcriptional activator of gluconeogenic genes Cat8p are essential for Q biosynthesis. However, the *coq7*/*cat5* null mutant fails to produce Q_6 (Fig. 1*D*), and instead accumulates HHB, the predominant intermediate found in the yeast Q mutants *coq3*-*coq8* (8, 11). This finding suggests that the inability to produce Q is a very specific defect and cannot be caused by a lack of glucose derepression in the examined mutant strains.

Gluconeogenic Derepression Is Defective in Both coq and atp2 Mutants—To determine whether a general loss of respiration affects glucose derepression of gluconeogenic genes, activation of a PCK1-lacZ reporter fusion containing the entire phosphoenolpyruvate carboxykinase promoter was assayed in a variety of respiratory yeast mutants. As shown in Table II, all of the wild-type strains used, although differing in absolute specific activities, reveal a dramatic increase in specific β-galactosidase activity upon the shift to nonfermentable growth conditions. In contrast, such induction is completely absent in the *coq7*/*cat5* mutant and in another Q-deficient mutant (*coq3*). A yeast strain containing a deletion in the *ATP2* gene (encoding the β subunit of the F_1-ATPase) also fails to activate the phosphoenolpyruvate carboxykinase promotor, although Q is still produced in this strain (11). The lack of ATPase activity causes a pleiotropic effect that results in a suppression of the bc_1 complex and a severe reduction of respiration (10). There is a similar lack of β-galactosidase activation in four other mutants with defects in respiration. The mutants either affect the respiratory chain in a structural component (Cox7p, Ref. 36), its synthesis (Cox10p, affecting heme a synthesis, Ref. 37), its assembly (Cox15p, Ref. 38), or in general mitochondrial gene expression (Mtf2, specifically needed in *COX1* expression but also affecting overall mitochondrial gene expression, Ref. 39). These results indicate that glucose derepression of gluconeogenic enzymes is dependent on intact respiratory metabolism.

TABLE II
Derepression of gluconeogenic PCK1-promoter is dependent on mitochondrial energy metabolism

Specific enzyme values were determined in repressed cells (4% glucose) or after derepression (3% ethanol) for 6 h in synthetic complete media lacking uracil. As a reporter the episomal PCK1-lacZ fusion pPEPCKlacZ was used.

Strain[a]	Relevant genotype	Specific β-galactosidase activity	
		Glucose	Ethanol
		units/mg	
CEN.PK2–1C	Wild type	11	5446
EG103	Wild type	8	3411
WAY.5–4A	Wild type	54	9462
CEN.MP3–1A	Δ*cat5*/Δ*coq7*	5	10
FW103	Δ*coq3*	7	19
DO103	Δ*atp2*	18	26
ENY.MP7–3C	*cox7–7*	63	165
ENY.MP30–4D	*mtf2–30*	53	168
ENY.MP44–3A	*cox15–44*	37	48
ENY.MP60–1C	*cox10–60*	46	82

[a] Yeast strains are described in Table I.

Rescue by Supplementation with Q_6—We tested whether respiration (as assayed by growth on ethanol) and derepression of gluconeogenic genes could be restored by exogenous Q. As shown in Fig. 2 the *coq7*/*cat5* mutant fails to grow on media containing a nonfermentable carbon source (3% ethanol). This is also true for another *coq* mutant, *coq3*Δ, and two respiratory mutants, *atp2*Δ, and *cor1*Δ (completely lacks the bc_1 complex, Ref. 40). This growth defect was corrected in the two *coq* mutants by supplementation with 15 μM Q_6, and after a brief lag compared with the wild-type strain, the rescued *coq7*/*cat5* and *coq3* strains grew to the same stationary cell titer. Neither the *atp2* nor the *cor1* mutants could be rescued by exogenously added Q_6. The effect of the addition of Q_6 on the derepression of gluconeogenesis was simultaneously investigated. Addition of Q_6 fully restored gluconeogenic enzyme activities in both the *coq7*/*cat5* mutant and the *coq3* mutant (Table III). However, such addition of Q_6 failed to restore induction of these activities in the *atp2* and *cor1* mutants. Addition of Q_6 also increased both the NADH dehydrogenase activity and the rate of oxygen consumption of the *coq* mutants; the latter showed a 2–3-fold increase from their average baseline in YPE alone of 10 μl of O_2/min/OD (data not shown). However, oxygen consumption

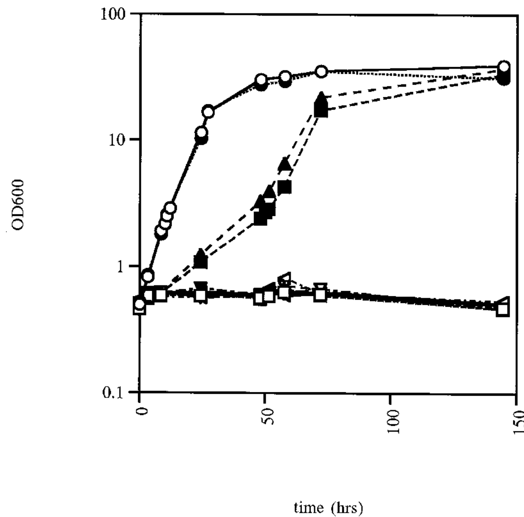

FIG. 2. **Exogenous Q_6 rescues the *coq7Δ/cat5Δ* yeast mutant.** Yeast strains W303.1B (wild-type: ○, ●), W303ΔCOQ7 (*coq7Δ/cat5Δ*; [squlo] ■), CC303 (*coq3Δ*: Δ, ▲), CC304 (*atp2Δ*: ▽, ▼), and W303ΔCOR1 (*cor1Δ*: ▲, ▲) were grown overnight in 15 ml of YPD to stationary phase and then diluted into 40 ml of YPE ($OD_{600\ nm}$ = 0.6) with (*filled symbols*) or without (*open symbols*) supplementing 15 μM Q_6. Growth was monitored by $OD_{600\ nm}$ measurements and at the same time samples were taken for enzymatic assays.

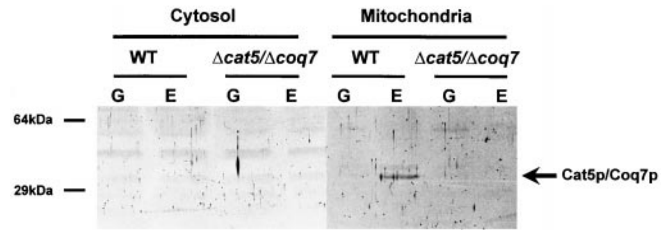

FIG. 3. **Nonfermentable growth conditions increase levels of Coq7p/Cat5p.** Crude extracts from wild-type (CEN.PK2–1C) and *coq7/cat5* mutants (CEN.MP3–1A) were fractionated into cytosolic and crude mitochondrial samples. Cells were grown under glucose repressed conditions (YPD = G) and derepressed by a shift to ethanol containing medium (YPE = E). Proteins (50 μg of cytosolic fractions and 10 μg of mitochondrial fractions per lane) were separated by SDS-PAGE in 12% polyacrylamide and subjected to immunoblot analysis using polyclonal anti-Cat5p antiserum.

TABLE III
Q_6 rescues gluconeogenic enzyme levels in coq mutants

Wild type (W303.1B) and mutant (W303.1B background) cells were grown as indicated in Fig. 2. PEP carboxykinase (PEPCK) and isocitrate lyase (ICL) were measured as key enzymes of gluconeogenesis.

Relevant genotype	Growth conditions	Specific activities	
		PEPCK	ICL
		milliunits/mg	
Wild type	YPD	16	9
	YPE, 12 h, OD_{600} = 3	37	110
	YPE, 27 h, OD_{600} = 17	48	51
	YPE + Q_6, 12 h, OD_{600} = 3	53	57
	YPE + Q_6, 27 h, OD_{600} = 17	72	50
Δcat5/Δcoq7	YPD	5	6
	YPE, 52 h, OD_{600} = 0.6	<1	3
	YPE, 72 h, OD_{600} = 0.6	<1	3
	YPE + Q_6, 52 h, OD_{600} = 3	112	114
	YPE + Q_6, 72 h, OD_{600} = 17	85	54
Δcoq3	YPD	17	4
	YPE, 48 h, OD_{600} = 0.6	9	5
	YPE, 72 h, OD_{600} = 0.6	10	4
	YPE + Q_6, 48 h, OD_{600} = 3	130	95
	YPE + Q_6, 72 h, OD_{600} = 22	37	47
atp2Δ	YPD	18	4
	YPE, 52 h, OD_{600} = 0.6	<1	1
	YPE, 72 h, OD_{600} = 0.7	1	1
	YPE + Q_6, 52 h, OD_{600} = 0.6	<1	1
	YPE + Q_6, 72 h, OD_{600} = 0.7	11	2
cor1Δ	YPD	7	6
	YPE, 52 h, OD_{600} = 0.6	<1	2
	YPE, 72 h, OD_{600} = 0.7	7	5
	YPE + Q_6, 52 h, OD_{600} = 0.7	<1	3
	YPE + Q_6, 72 h, OD_{600} = 0.6	15	5

rates in the *atp2* and *cor1* mutants remained very low in the presence or absence of exogenous Q (baseline rates in YPE and YPE + Q_6 were approximately 4 μl of O_2/min/OD, data not shown). These data show that the loss of Cat5p/Coq7p indirectly influences induction of gluconeogenesis, and the defect can be completely suppressed by the addition of Q_6.

Localization of Coq7p/Cat5 to the Mitochondria—To determine the localization of Coq7p/Cat5p in yeast, we used a polyclonal antibody against Coq7p/Cat5p. As shown in Fig. 3, the specific immunodetection of the protein was possible exclusively in crude mitochondrial fractions of wild-type cells grown under nonfermentable conditions. Since Coq7p/Cat5p was not detectable in glucose-repressed cells (Fig. 3), we assume that most of the protein was synthesized during the transition from fermentative to respiratory growth. As judged by the mobility in SDS-PAGE, no deviation from the predicted molecular mass (31 kDa) was obtained. Further subcellular localization was performed by employing a fusion of the *COQ7* gene and the sequence coding for an epitope peptide from the hemagglutinin viral protein. Western analysis of yeast subcellular fractions indicates that the Coq7p-HA fusion protein cofractionates with the mitochondria (Fig. 4). The lack of mitochondrial contamination in the cytosol was verified with the mitochondrial marker $F_1\beta$-ATPase (41). The crude mitochondrial fractions depicted in Figs. 3 and 4 contained some contaminating organelles, detected with antibodies to Sec62p (endoplasmic reticulum, Ref. 42), Kex2p (Golgi, Ref. 43), and ALPp (vacuole, Ref. 44) (Fig. 5). When the mitochondria were further purified over a Nycodenz gradient (33), the abundance of all of the contaminating proteins dropped dramatically or disappeared altogether (Fig. 5), indicating that Coq7p cofractionates with the mitochondria.

Localization of Coq7p/Cat5p to the Inner Mitochondrial Membrane—To determine the submitochondrial localization of Coq7p, purified yeast mitochondria were subjected to various treatments which break apart the mitochondria and allow the isolation of proteins from different compartments (45). The outer membrane was disrupted through osmotic swelling, thus releasing the soluble proteins of the intermembrane space, but leaving the inner membrane intact. Western analysis verified that Coq7p was absent from the intermembrane space fraction as compared with the marker cytochrome b_2 (data not shown). Peripherally bound and soluble proteins were extracted from the resulting mitoplasts and disrupted membranes and were treated with either alkaline carbonate or urea. Both the alkaline carbonate and the urea treatments extract both matrix and peripherally bound membrane proteins which remain in the supernatant following high speed centrifugation (46, 47). As shown in Fig. 6, Coq7p fractionated in a manner similar to cytochrome c_1, an integral inner membrane protein (48). Conversely, two soluble matrix proteins, Mas2 and Hsp60 (49, 50), and peripheral inner membrane proteins such as $F_1\beta$-ATPase were released into the supernatant (Fig. 6, *B-D*). OM45, an outer membrane protein with one transmembrane domain (51), was also extracted from the pellet fraction (Fig. 6*F*). In contrast, Coq7p remained solely in the pellet with both extraction

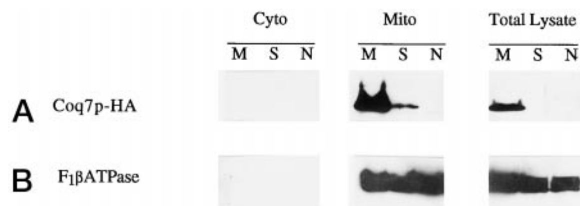

FIG. 4. **Coq7-HA protein localizes to a mitochondrial fraction.** Yeast cells (JM43ΔCOQ7) were transformed with a single copy plasmid, psHA71 (*S*), or a multiple copy plasmid, pmHA71 (*M*), expressing the COQ7 protein with a carboxyl-terminal epitope tag from the hemagglutinin viral protein. The pNMQ71 plasmid containing only the COQ7 nucleotide sequence was also transformed into this strain (*N*). Yeast cells containing these plasmids were grown to saturation, and the cells were collected and lysed and fractionated by standard methods (see "Experimental Procedures"). 50 μg of protein from both the cytosolic and mitochondrial fractions and the total lysate was analyzed by SDS-PAGE. The gel was transferred for Western analysis by chemiluminescence detection using purified 12CA5 antibodies to the HA-epitope tag (*A*) and antibodies to the mitochondrial protein $F_1\beta$-ATPase (*B*).

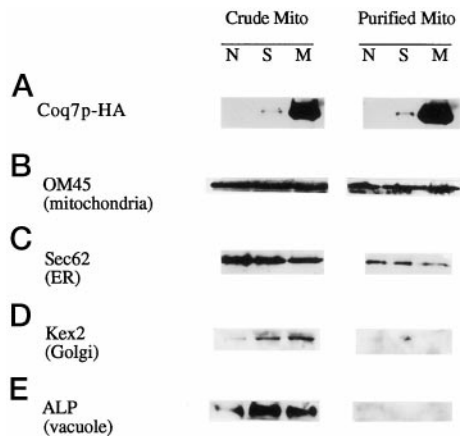

FIG. 5. **Coq7p/Cat5p co-fractionates with purified mitochondria.** Crude mitochondrial preps of yeast cells (JM43ΔCOQ7) transformed with psHA71 (*S*), pmHA71 (*M*), and pNMQ71 (*N*) were purified over Nycodenz gradients (see "Experimental Procedures"). *Panel A,* 50 μg of protein from crude and purified mitochondrial preparations was electrophoresed by SDS-PAGE and the subsequent gel transferred for Western analysis by chemiluminescence detection using purified 12CA5 antibodies to the HA-epitope tag. *Panels B-E,* 30 μg of protein from crude and purified mitochondrial preparations were electrophoresed and blotted as above using purified antibodies to the OM45 outer mitochondrial membrane protein (*Panel B*), Sec62 (*Panel C*), Kex2 (*Panel D*), or alkaline phosphatase (*Panel E*).

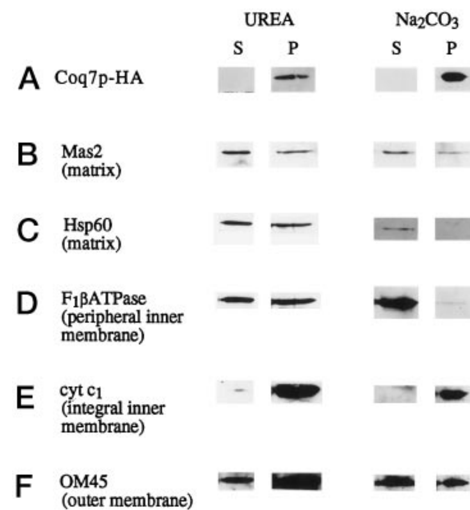

FIG. 6. **Coq7p is an integral inner membrane protein.** Mitoplasts prepared from JM43ΔCOQ7:pmHA71 were either treated with 2 M urea or 0.1 M Na_2CO_3, pH 11.5, incubated on ice, and centrifuged as described (see "Experimental Procedures"). 2 μg of protein from each resultant supernatant (*S*) and pellet (*P*) fraction were analyzed by SDS-PAGE, transferred for Western analysis, and probed via chemiluminescence detection using antibodies to the HA-epitope tag (*A*), Mas2 (*B*), Hsp60 (*C*), $F_1\beta$-ATPase (*D*), cytochrome c_1 (*E*), and OM45 (*F*).

protocols (Fig. 6*A*), colocalizing with cytochrome c_1 (Fig. 6*E*). Therefore, Coq7p is an inner mitochondrial membrane protein.

DISCUSSION

This study provides evidence for the direct involvement of Coq7p/Cat5p in Q biosynthesis. As shown in Fig. 2 the growth defect of a *coq7/cat5* and a *coq3* mutant under nonfermentable conditions can be restored by external feeding with 15 μM Q_6. Moreover, addition of Q_6 also restores the ability to activate gluconeogenic (phosphoenolpyruvate carboxykinase and isocitrate lyase) enzymes during the transition from glucose to ethanol metabolism (Table III). Such gluconeogenic gene activation is absent in other mutants affecting Q biosynthesis (*coq3*Δ) as well as in a broad range of respiratory deficient yeast strains (*atp2*Δ*, cox7-7, mtf2-30, cox15-44,* and *cox10-60*) as seen by a lack of derepression of gluconeogenic promoters (Table II). This indicates that glucose derepression is dependent on intact respiratory metabolism. Furthermore, our results indicate that while the glucose derepression system influences Q biosynthesis, this regulatory system is not essential for production of Q. It is known that high levels of glucose repress yeast Q biosynthesis (11, 52), and *COQ3* and *COQ7/CAT5* mRNA levels (8, 53). The expression of a *COQ7/CAT5-lacZ* fusion gene is repressed 5–6-fold by glucose (7). Moreover, Coq7p/Cat5p is not detectable in glucose-repressed cells and is mainly synthesized after the transfer of cells to nonfermentable growth conditions (YPE) (Fig. 4). Many nuclear-encoded mitochondrial proteins are regulated by carbon source, although there can be significant variation depending on the strain used (54). While the *cat5/coq7* mutant fails to produce Q, other *cat* mutants (*cat1, cat3,* and *cat8*) with defects in glucose derepression produce Q (Fig. 1). Thus neither the pleiotropic Cat1p/Cat3p (Snf1p/Snf4p) protein kinase involved in glucose derepression nor the transcriptional activator of gluconeogenic genes, Cat8p (20, 23), are essential for Q biosynthesis. This finding supports the idea that the inability to produce Q is a very specific defect (11) and cannot be caused by a lack of glucose derepression in the examined mutant strains.

Western analysis of cell fractions indicates that both Coq7p and the fusion protein Coq7p-HA cofractionate with mitochondria (Figs. 3 and 4). The lack of contaminating organelles in the pure mitochondrial preparations provides compelling evidence for a mitochondrial location of Coq7p (Fig. 5). Two other yeast polypeptides (Coq3p and Coq5p) required for the respective *O*-methylation and C-methylation steps of Q biosynthesis are also located in the mitochondria (24, 55, 56). The amino terminus of each of these latter polypeptides has a typical mitochondrial leader sequence, including the presence of several basic residues, an absence of acidic residues, and a sequence consistent with the tendency to form amphipathic α-helices (57). A 3-amino acid consensus motif common to mitochondiral matrix proteins (58) is present in the amino termini of Coq2p (59), Coq3p (55), Coq5p (24, 56), Coq4p, Coq6p, and Coq8p.[2] The amino terminus of Coq7p contains neither a typical leader sequence nor the matrix motifs and even has two acidic residues in its N-terminal region. However, unlike the matrix proteins Coq3p and Coq5p, Coq7p is instead located in the inner mitochondrial membrane, and cofractionates with cytochrome c_1, an integral inner membrane protein (Fig. 6). An

[2] A. Y. Hsu, P. T. Lee, and C. F. Clarke, unpublished data.

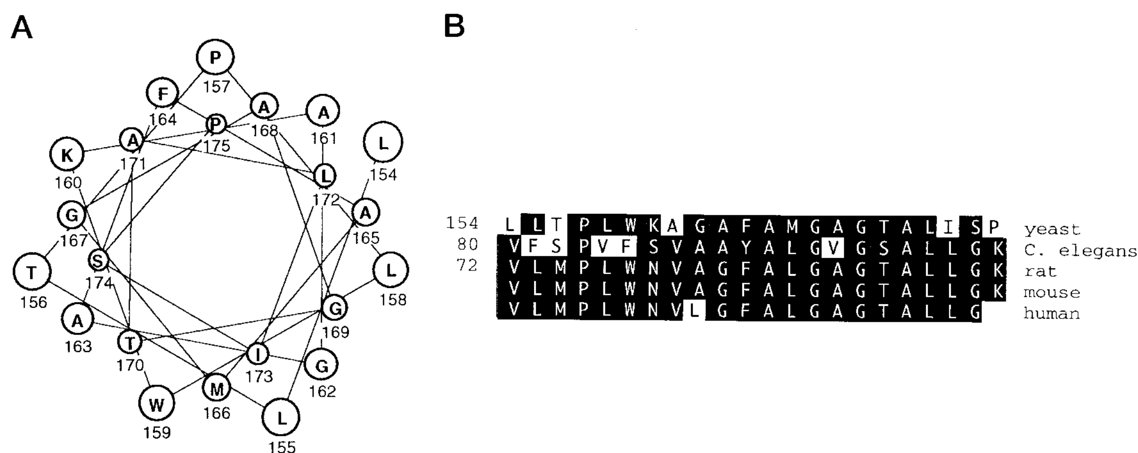

FIG. 7. **Potential membrane insertion region forms an α-helical wheel.** *A*, an α-helical wheel representation of residues 154–175 of yeast Coq7p analyzed through the program PROTEAN. *B*, potential membrane insertion regions present in the *C. elegans*, rat, mouse, and human Coq7 homologs show high identity with the yeast protein. GenBank accession numbers are as follows: *S. cerevisiae*, X82930; *C. elegans*, U13642; rat, U46149; mouse, U81277; human, U81276. Mouse and human amino acid residues are not numbered since only partial sequences are available. Previous ambiguities in the rat sequence noted by Ewbank *et al.* (6) have been resolved and the results deposited in GenBank (U46149).

absence of such targeting motifs is not uncommon to inner mitochondrial membrane proteins. There is a class of such inner membrane proteins which have no cleavable targeting sequence, and likely contain internal targeting sequences (60) most of which have yet to be characterized (61).

The amino acid sequence of yeast Coq7p does contain a region (residues 154–175) predicted to be in an [alpha[-helical conformation (Fig. 7*A*), with the ability to insert into the membrane as determined by the MOMENT program (62, 63). The amphipathic nature of this predicted membrane helix in other systems leads to membrane protein association via charged pairs (64, 65). The orientation of the NH_2- and COOH-terminal regions has yet to be experimentally determined. However, the abundance of positively charged residues NH_2-terminal to the potential membrane insertion element of Coq7p indicates a matrix localization of this portion of the protein, as determined by the program PSORT (66). Interestingly, this program predicts Coq3p and Coq5p to be mitochondrial matrix proteins, a prediction that has been confirmed experimentally (24, 55, 56).

Similar potential membrane insertion regions are also present in the *C. elegans*, rat, mouse, and human Coq7 homologs (Fig. 7*B*). These homologs have a high degree of identity throughout the entire protein; in the region between amino acids 92 and 272 of the yeast sequence, yeast and *C. elegans* are 41% identical, yeast and rat are 47% identical, and *C. elegans* and rat are 57% identical. Since *C. elegans clk-1* (6) and the rat *COQ7* (12) both complemented yeast *coq7/cat5* mutants, it is evident that these proteins share the same function and location. The location of Coq7/Cat5 is consistent with its proposed role in aiding the conversion of HHB to Q (8); however, its specific function in the production of Q is not known. It is interesting to note that HHB is the predominant intermediate found in each of the yeast Q mutants *coq3-coq8* (11). One possibility is that Coq7p serves to anchor a multisubunit complex composed of one or more of the Coq proteins to the inner membrane, thus facilitating their ability to act on the lipophilic Q intermediates.

Given the functional conservation of yeast, rat, and *C. elegans* Coq7p/Cat5p/Clk-1p, yeast provide a suitable model to unravel the action of this protein in aging and delayed development. Although results shown here identify *COQ7* as a gene involved in Q biosynthesis, we have not ruled out the possibility of an unknown secondary function responsible for the *C. elegans clk-1* mutant phenotype. It seems unlikely that the characterized *clk-1* mutations that effect increased longevity in *C. elegans* result in a complete loss of Q and respiratory function, since the recovery of these mutant alleles represented a rare event (4). In yeast, respiratory defective mutants arise at a high frequency due to the large number of nuclear genes required to produce respiratory competent mitochondria (10). In addition, yeast ρ^- mutants (lacking mitochondrial respiration) are reported to have shorter life spans (67). Instead, it seems more likely that the mutations in *clk-1* and the resulting effects on life span and development in *C. elegans* may relate to changes in the amount of Q. In this event, the abundance of Q may influence the extent to which oxidants are generated by mitochondrial respiration (68, 69). Many *in vitro* studies implicate ubisemiquinone ($Q^{\cdot}$) as the major site of electron leakage (70, 71). Accordingly, a decrease in Q in *clk-1* mutants could decrease respiratory electron transfer and perhaps the generation of superoxide, hydrogen peroxide, and hydroxyl radicals that have been proposed to contribute to cellular aging (72, 73). In this oxidative stress theory of aging, mitochondria are considered to be both the main source and the target of oxygen-derived free radicals (72). However, the sites of superoxide and hydrogen peroxide generation *in vivo* is still an open question. The *in vitro* studies employ conditions that enhance the propensity of radical production. For example, drugs such as antimycin which enhance superoxide production, modify the interaction of $Q^{\cdot}$ with proteins, alter the stability of $Q^{\cdot}$, and elicit superoxide production at sites which *in vivo* may play a very minor role (74, 75). Hence the influence of Q levels on both the pro- and antioxidant activities of the mitochondrial respiratory chain remains to be determined (76–78). Reduced Q (QH_2) is capable of acting as a lipid-soluble antioxidant, scavenging radicals both directly, in a manner similar to that of vitamin E, and indirectly, by regenerating vitamin E (79–81). Q has been shown to be a functional antioxidant in yeast under conditions promoting lipid peroxidation (82). Q in the plasma membrane also plays a role in extracellular ascorbate stabilization (83). In view of this potential balance of antioxidant and pro-oxidant activities of Q, it will be very important to determine the effect of the *clk-1* mutations on levels and intracellular distribution of Q, cell cycle length, and life extension in the yeast model.

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