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CYC2 Encodes a Factor Involved in Mitochondrial Import of Yeast Cytochrome *c*

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The gene *CYC2* from the yeast *Saccharomyces cerevisiae* was previously shown to affect levels of mitochondrial cytochrome *c* by acting at a posttranslational step in cytochrome *c* biosynthesis. We report here the cloning and identification of the *CYC2* gene product as a protein involved in import of cytochrome *c* into mitochondria. *CYC2* encodes a 168-amino-acid open reading frame with at least two potential transmembrane segments. Antibodies against a synthetic peptide corresponding to the carboxyl terminus of the predicted sequence were raised. These antibodies recognize multiple bands on immunoblots of mitochondrial extracts. The intensities of these bands vary according to the gene dosage of *CYC2* in various isogenic strains. Immunoblotting of subcellular fractions suggests that the *CYC2* gene product is a mitochondrial protein. Deletion of *CYC2* leads to accumulation of apocytochrome *c* in the cytoplasm. However, strains with deletions of this gene still import low levels of cytochrome *c* into mitochondria. The effects of *cyc2* mutations are more pronounced in *rho*$^-$ strains than in *rho*$^+$ strains, even though *rho*$^-$ strains that are *CYC2*$^+$ contain normal levels of holocytochrome *c*. *cyc2* mutations affect levels of iso-1-cytochrome *c* more than they do levels of iso-2-cytochrome *c*, apparently because of the greater susceptibility of apo-iso-1-cytochrome *c* to degradation in the cytoplasm. We propose that *CYC2* encodes a factor that increases the efficiency of cytochrome *c* import into mitochondria.

The yeast *Saccharomyces cerevisiae* contains two forms of cytochrome *c*, iso-1-cytochrome *c* and iso-2-cytochrome *c* (44). These two isozymes are encoded by the *CYC1* and *CYC7* genes and constitute, respectively, 95 and 5% of the total complement of cytochrome *c* in aerobically grown cells. Numerous mutants specifically deficient in one or both isocytochromes *c*, but maintaining normal levels of other mitochondrial cytochromes, have been isolated by the following three methods: the spectroscopic scanning procedure, based on the diminished Cα absorption band in intact cells (36); the benzidine staining procedure, based on the diminished peroxidative activity of cytochrome *c* (5, 42); and the chlorolactate procedure, based on resistance to chlorolactate and diminished growth on lactate medium (41). Genetic analyses revealed that mutations of only three genes resulted in greater than 50% deficiencies of cytochrome *c* without affecting other mitochondrial cytochromes: (i) certain mutations of *CYC1* resulted in complete deficiency of iso-1-cytochrome *c* without affecting the normal 5% level of iso-2-cytochrome *c*; (ii) certain mutations of *CYC3*, which encodes cytochrome *c* heme lyase (also called holocytochrome *c* synthase, EC 4.4.1.17), the enzyme catalyzing covalent attachment of heme to apocytochrome *c* (8, 9), resulted in complete deficiency of both iso-1- and iso-2-cytochrome *c*; and (iii) mutations of *CYC2*, which are the subject of this paper, resulted in substantial reductions in levels of total cytochrome *c*. However, no *cyc2* mutations, of the large number that have been isolated, reduce cellular levels of cytochrome *c* to less than 10% of normal. Previous genetic analyses revealed *CYC2* to be a relatively small gene (29) on the left arm of chromosome XV (31).

Cytochrome *c* is synthesized in the cytoplasm, from nuclear-encoded message, and then transported across the outer mitochondrial membrane by a mechanism that appears to be distinct from that used by at least some other imported mitochondrial proteins (7, 12, 15). This paper describes the molecular cloning and DNA sequencing of *CYC2* and presents evidence that the *CYC2* gene product is a mitochondrial protein involved in import of cytochrome *c* into mitochondria.

MATERIALS AND METHODS

Genetic nomenclature. The gene symbol *CYC* denotes loci that significantly affect cytochrome *c*. *CYC1*, *CYC7*, and *CYC3* encode, respectively, iso-1-cytochrome *c*, iso-2-cytochrome *c*, and cytochrome *c* heme lyase. Mutant alleles are presented in lowercase followed by the allele number, e.g., *cyc7-67*. *CYC7-H3* denotes a mutant that produces high levels of normal iso-2-cytochrome *c* because of a deletion in the promoter region of *CYC7* (24).

Cloning and genetic analyses. Standard techniques were employed for culturing and genetic manipulation of *Escherichia coli* (33) and *S. cerevisiae* (38). The procedures used for analysis of mutations involving cytochrome *c* have also been described previously (39, 41).

Strain B-7553 was constructed by transforming (25) strain B-6748 (*MAT***a** *cyc1-738::lacZ cyc7-Δ::CYH2 ura3-52 his3-Δ1 leu2-3 leu2-112 trp1-289 cyh2*) (17) with plasmid pAB293 containing the *CYC1*$^+$ gene and a *URA3*$^+$ marker. Strains lacking the plasmid sequences and the original *cyc1* deletion were selected with 5-fluoroorotic acid, leaving just the *CYC1*$^+$ sequences, as confirmed by Southern blot analysis. B-7908 was constructed by transforming B-6748 with plasmid pAB595 containing the *CYC7-H3* gene as described previously (7). B-8132 and B-8133 were constructed by transforming B-7553 and B-7908, respectively, with plasmid

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pJS3 containing a *cyc2*::*LEU2*$^+$ disrupter (see Fig. 1C and Results). The presence of *LEU2*$^+$ at the *CYC2* locus was confirmed by polymerase chain reaction.

Strains B-7553, B-7908, B-8132, and B-8133 were converted to *rho*$^-$, creating strains B-8139, B-8140, B-8141, and B-8142, respectively, by being cultured on a YPD plate containing a gradient of ethidium bromide (100 μl of a 10-mg/ml ethidium bromide solution applied to an 11.5-mm-diameter filter disk in the center of the plate). Colonies were picked from the edge of the region of growth inhibition. Conversion to *rho*$^-$ was confirmed by low-temperature spectroscopic examination of whole cells and by crossing to tester strain B-1999 (*rho*$^-$ *MAT*α *CYC1*$^+$ *his1-1 lys2-1 trp2-1*).

Strain B-8033 was constructed by transforming strain B-7503 (*MAT***a** *CYC1*$^+$ *CYC7*$^+$ *his5-2 leu2-3 leu2-112 ura3-52*) with the multicopy plasmid pBC58 containing *CYC2* (see below).

Subcellular fractionation and immunoblotting. Cytoplasmic and mitochondrial fractions were isolated as described previously (7), except that mitochondria were isolated in a solution containing 0.5 M mannitol, 50 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH, and 1 mM EDTA (pH 7.4) and the *rho*$^-$ mitochondria were pelleted at 27,000 × *g* for 15 min. A combination of minimal treatment with zymolyase, gentle homogenization during cell lysis, and avoidance of repeated freeze-thaw cycles of samples frozen in sodium dodecyl sulfate (SDS) loading buffer appeared to be important for preventing degradation of apocytochrome *c* and heme lyase. Where indicated, samples were treated with 0.35 mg of proteinase K per ml in the presence or absence of 0.5% Triton X-100 and then inhibited with phenylmethylsulfonyl fluoride as previously described (7). Immunoblotting with anti-cytochrome *c* antibodies was also carried out as previously described (7), except that the antibodies were affinity purified with commercially available cytochrome *c*-agarose (Sigma Chemical Co.) and the blots were visualized with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and tetramethylbenzidine (Kierkegaard and Perry Laboratories) as a substrate (see Fig. 6 and 7).

Anti-*CYC2* antibodies were produced in rabbits by using a synthetic peptide (CKEGWNSDNVYKLS) corresponding to the predicted COOH-terminal of the *CYC2* protein plus an extra cysteine residue at the amino terminus for use in coupling to carrier. Conjugation to keyhole limpet hemocyanin via the reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (13) and immunization were performed by the Berkeley Antibody Company with an initial immunization with 500 μg of conjugate followed by booster immunizations of 250 μg. Rabbit sera were affinity purified with synthetic peptide coupled to a Sulfo-link column (Pierce Chemical Co.) as instructed in the manufacturer's instructions. Immunoblots with the anti-*CYC2* antibodies were stained with either biotinylated goat anti-rabbit secondary antibody and the ABC biotin-avidin reagent (Vector Laboratories) with 4-chloronaphthol as a substrate (see Fig. 4) or a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody with methylbenzidine as a substrate (see Fig. 5).

The following were used to monitor the distribution of marker enzymes in subcellular fractionations. Anti-glucose-6-phosphate dehydrogenase antibodies were obtained from Sigma. Anti-cytochrome b_2 antibodies were a generous gift of Richard Hallberg of Syracuse University. Antibodies recognizing cytochrome *c* heme lyase have been described previously (7). Relative levels of marker proteins in different fractions were determined by parallel immunoblotting of dilutions of each fraction. Enzyme activity of cytochrome b_2 was measured as previously described (7).

Determination of cytochrome *c* contents. Cytochrome *c* levels in intact cells were estimated by using a Hartree low-dispersion spectroscope at −196°C (40). More-accurate determinations were performed by low-temperature (−196°C) spectrophotometric recording with a modified Aviv model 14DS spectrophotometer as described by Hickey et al. (16).

RESULTS

Molecular cloning and DNA sequencing of *CYC2*. The wild-type *CYC2* gene was cloned by complementation of the recessive marker *cyc2-3* in the strain JB26-1D (*MAT***a** *cyc2-3 cyc1-345-F ura3-52 leu2-3 leu2-112 trp1-289 arg4-17*). The *cyc1-345-F* allele diminishes iso-1-cytochrome *c* to approximately 30% of the normal level because of the introduction of an internal initiator codon (43). Use of this allele facilitated identification of *CYC2*$^+$ and *cyc2*$^-$ strains by growth on lactate medium, since *cyc1-345-F CYC2*$^+$ strains grow distinctly better than *cyc1-345-F cyc2*$^-$ strains, whereas the difference is less marked in comparing *CYC1*$^+$ *CYC2*$^+$ with *CYC1*$^+$ *cyc2*$^-$ strains. Strain JB26-1D was transformed with the two yeast genomic banks described, respectively, by Nasmyth and Reed (27) and Carlson and Botstein (1). Transformants, selected as either Trp$^+$ or Ura$^+$, respectively, were suspended in sterile water and plated on lactate medium, which selects for strains containing *CYC2*$^+$ and *CYC1*$^+$ plasmids. The *CYC1*$^+$ and *CYC2*$^+$ transformants were distinguished by crossing them to strains JB10-17D (*MAT*α *cyc2-3 his3-Δ1 trp1-289*) and KZ177-4D (*MAT*α *cyc1-1 ura1-1*) and examining the cytochrome *c* content of the resulting diploids. A total of nine potential *CYC2* transformants were recovered by these procedures. Restriction mapping of four of these plasmids with *Hin*dIII revealed common restriction fragments, thus indicating that they all contained a common segment of chromosomal DNA. One of these plasmids, pBC58, was chosen for further study.

The insert in pBC58 was shown by genetic analysis to contain a segment of DNA corresponding to the *CYC2* chromosomal locus. The *Hin*dIII fragment from pBC58 was transferred to the *URA3* integrating plasmid YIp5. Following cleavage of this plasmid at a *Kpn*I site within the *Hin*dIII fragment, it was used to transform the *ura3-52 CYC2*$^+$ strain B-7034. A Ura$^+$ transformant was crossed to a *ura3*$^-$ *cyc2*$^-$ strain. Because no complete tetrads were obtained from this cross, one of the Ura$^+$ progeny was recrossed to the *ura3*$^-$ *cyc2*$^-$ strain. In this second cross, the PD/NPD/T ratio of 11:0:0 for Ura$^+$/Ura$^-$ and Cyc$^+$/Cyc$^-$ established that the plasmid integrated at the *CYC2* locus and, thus, that the plasmid contained DNA from this locus.

The portions of the insert in pBC58 corresponding to *CYC2* were identified both by deleting *Hin*dIII fragments from pBC58 and by transferring segments to plasmid YEp24 (28). Strain JB26-1D (*MAT***a** *cyc2-3 cyc1-345-F ura3-52 trp1-289 arg4-17 leu2-3 leu2-112*) was transformed with the pBC58 or YEp24 derivatives; the transformants were crossed to JB16-19C (*MAT*α *cyc2-3 ura3-52 leu2 lys2*), and the cytochrome *c* contents of the resultant diploids were determined by low-temperature (−196°C) spectroscopic examination of intact cells. Lack of complementation of the *cyc2*$^-$ mutant indicated that the plasmid lacked at least part of *CYC2*. The results, summarized in Fig. 1A, indicated that

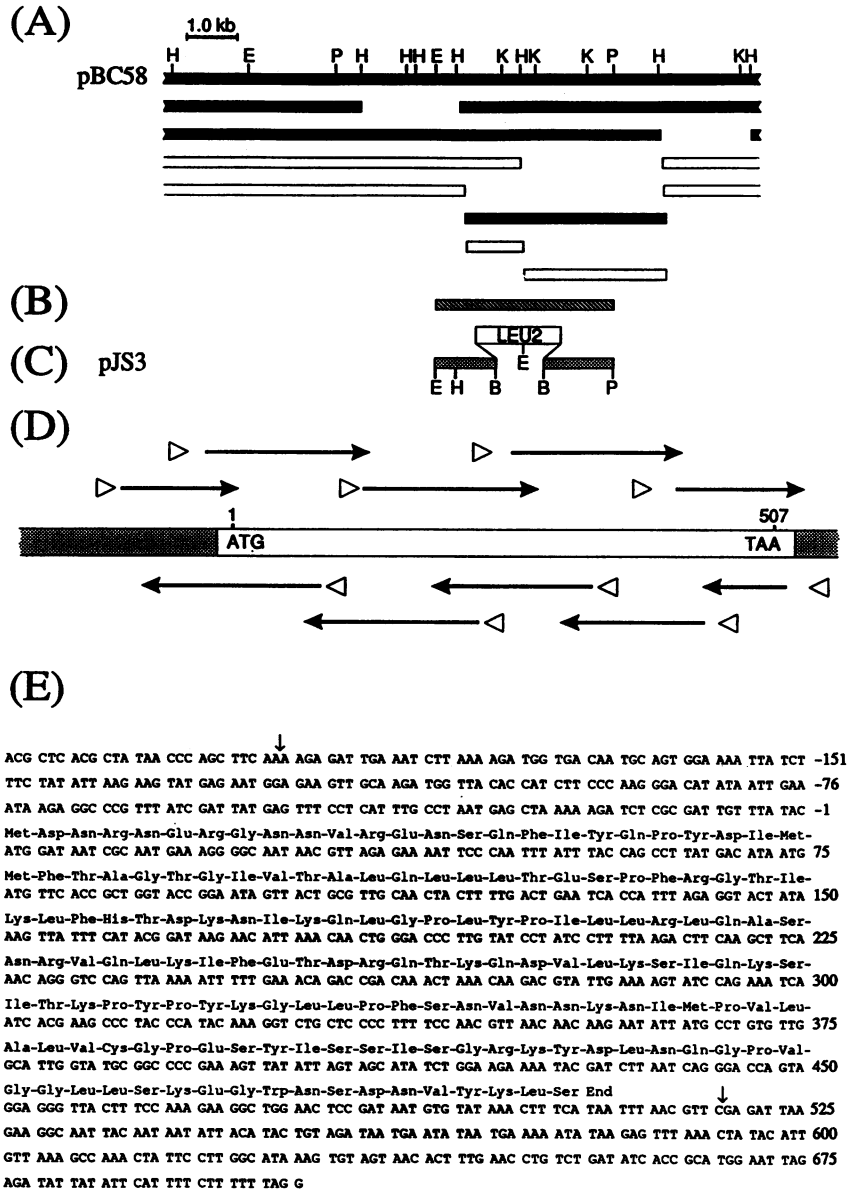

FIG. 1. (A) Region of the yeast genome containing *CYC2*. The pBC58 insert is shown in the top line, with restriction sites for *Hin*dIII (H), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), and *Bam*HI (B). The next four lines depict pBC58 derivatives with deleted *Hin*dIII fragments. The sixth through eighth lines depict segments transferred from pBC58 to YEp24. Strain J26-1D (*cyc2*$^-$ *cyc1-345-F*) was transformed with the eight plasmids, the transformants were crossed to JB16-19C (*cyc2*$^-$ *CYC1*$^+$), and the cytochrome *c* contents of the diploids were estimated. The plasmids complementing *cyc2*$^-$ and therefore containing *CYC2* are denoted with filled-in bars, whereas the noncomplementing plasmids are devoted by open bars. (B) Cross-hatched bar denotes the *Eco*RI-*Pst*I segment used for sequencing. (C) Insert in plasmid pJS3 used for gene replacement of *CYC2* with *LEU2* (see text). (D) *CYC2* sequencing strategy. Preliminary sequence information was obtained with short *Hin*dIII-*Kpn*I and *Hin*dIII-*Hin*dIII fragments. Overlapping sequences of both strands (arrows) were obtained with custom-made oligonucleotides (open triangles). (E) DNA sequence of the *CYC2* gene and the deduced amino acid sequence. The numbers at the right refer to nucleotide positions, with the A of the ATG initiator codon assigned as number 1. The numbers to the left refer to amino acid positions. A possible TATA sequence can be found around position −83, and a possible TATATA transcriptional termination signal (32) can be found around position 683. The Met-Asp terminus is predicted to be acetylated (26). Two *Bam*HI sites (GGATCC) were inserted by site-directed mutagenesis between nucleotide −200 and −199 and between 517 and 518, as indicated by arrows. Plasmid pJS3 was constructed by replacing *CYC2* encompassed by these *Bam*HI sites with *LEU2* (C).

all or part of *CYC2* was in the *Eco*RI-*Pst*I segment, which was subsequently sequenced.

The *CYC2* sequence. Preliminary sequence information was obtained from the short *Kpn*I-*Hin*dIII segments (Fig. 1A) which were inserted into bacteriophages M13mp18 and M13mp19. The final sequences of both strands were achieved by sequencing of double-stranded plasmid DNA with Sequenase (United States Biochemical), with thermal denaturation, according to the manufacturer's directions, by priming with the oligonucleotides shown in Fig. 1D. Only

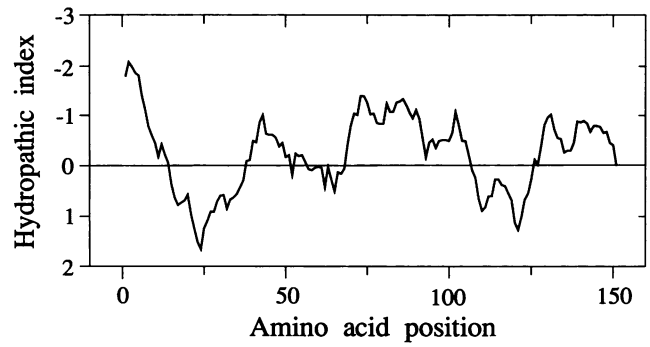

FIG. 2. Hydrophobicity profile for the predicted sequence of the *CYC2* gene product. The hydropathicity index was calculated by using the algorithm of Kyte and Doolittle (19) and the University of Wisconsin GCG program (4) with a window of 19 amino acid residues. Positive numbers correspond to hydrophobic segments.

TABLE 1. Amounts of holo-iso-1- or holo-iso-2-cytochrome *c* in *cyc2*-Δ *rho*$^+$ and *rho*$^-$ strains

Strain[a]	Designation (Fig. 3)	Pertinent genotype	Approx % holocytochrome *c* level[b]
B-7553	A	*rho*$^+$ *CYC1*$^+$ *cyc7*-Δ *CYC2*$^+$	100
B-8132	B	*rho*$^+$ *CYC1*$^+$ *cyc7*-Δ *cyc2*-Δ	20
B-8139	E	*rho*$^-$ *CYC1*$^+$ *cyc7*-Δ *CYC2*$^+$	100
B-8141	F	*rho*$^-$ *CYC1*$^+$ *cyc7*-Δ *cyc2*-Δ	10
B-7908	C	*rho*$^+$ *cyc1*-Δ *CYC7-H3 CYC2*$^+$	100
B-8133	D	*rho*$^+$ *cyc1*-Δ *CYC7-H3 cyc2*-Δ	50
B-8140	G	*rho*$^-$ *cyc1*-Δ *CYC7-H3 CYC2*$^+$	130
B-8142	H	*rho*$^-$ *cyc1*-Δ *CYC7-H3 cyc2*-Δ	20

[a] A series of isogenic strains was produced from strain B-6748 (*rho*$^+$ *CYC2*$^+$ *cyc1*-Δ *cyc7*-Δ) as described in Materials and Methods.

[b] Holo-iso-1-cytochrome *c* was measured for the top four strains, while holo-iso-2-cytochrome *c* was measured for the bottom four strains.

one significant open reading frame, shown in Fig. 1E, was present in the deduced DNA sequence. This 168-amino-acid sequence showed no homology or similarity to any sequence in the GenBank or to the data base of Mark Goebl (12a). The hydrophobicity profile of the predicted amino acid sequence presented in Fig. 2 suggests that the *CYC2* protein contains one or two transmembrane segments. The amino terminus of the predicted sequence is not typical of mitochondrial targeting signals, as it is hydrophilic and contains multiple acidic amino acid residues. In view of the apparent mitochondrial localization of the protein (see below), it should be noted that neither cytochrome *c* itself nor the *CYC3* gene product, encoding cytochrome *c* heme lyase, contains typical mitochondrial targeting signals and that at least one other imported mitochondrial protein with an acidic amino terminus is known (46).

For preliminary analysis of the *CYC2* transcription unit, a nitrocellulose filter containing RNA prepared from the normal *CYC2*$^+$ strain D311-3A and the *cyc2*$^-$ mutant strain B-4754 (*MATα cyc2-28* (UAG) *cyc1-1 CYC7-H1 trp1-1*) was probed with a nick-translated *Eco*RV-*Cla*I segment, which encompassed a portion of the *CYC2* gene. The single *CYC2* transcript was slightly longer than the transcript from the *CYC7* gene (probed in adjacent lanes on the same blot), which encodes a 112-amino-acid protein. The level of the *CYC2* mRNA was diminished in the *cyc2-28* strain, confirming the correspondence between the *Eco*RV-*Cla*I probe and the *CYC2* mRNA (data not presented).

Phenotype of the deleted *CYC2* strain. Strains with complete *CYC2* deletions were constructed by first performing site-directed mutagenesis (18) on the *Eco*RI-*Pst*I fragment encompassing the *CYC2* locus to introduce two *Bam*HI sites flanking the *CYC2* gene. These two sites allowed the convenient replacement of the *LEU2* gene, also flanked by *Bam*HI sites, for the coding region of *CYC2* to create plasmid pJS3 (Fig. 1C). Chromosomal deletions could be generated by transforming *leu2*$^-$ strains with a *Hin*dIII-*Pst*I fragment containing the *cyc2*::*LEU2* replacement flanked by homologous segments and selecting for Leu$^+$ transformants. Examination of cytochrome *c* content revealed that the *cyc2*-Δ strains were phenotypically similar to numerous previously isolated *cyc2* point mutants that retained approximately 20% of the normal level of total cytochrome *c*.

Iso-1- and iso-2-cytochrome *c* deficiencies in *rho*$^+$ and *rho*$^-$ strains. Although normal *CYC2*$^+$ strains contain approximately 95 and 5%, respectively, of iso-1- and iso-2-cytochrome *c*, most of the residual amount of holocytochrome *c* in *cyc2*$^-$ strains was composed of iso-2-cytochrome *c* (23). Furthermore, the total holocytochrome *c* content was further diminished in *rho*$^-$ *cyc2*$^-$ strains compared to *rho*$^+$ *cyc2*$^-$ strains (36). These relationships were systematically investigated with the series of isogenic strains (Table 1; Fig. 3). The results clearly show that the *cyc2*-Δ defect diminishes iso-1-cytochrome *c* more strongly than iso-2-cytochrome *c* and that this diminution is more pronounced in *rho*$^-$ strains.

The *CYC2* gene product is a mitochondrial protein. A synthetic peptide corresponding to the carboxyl terminus of the predicted *CYC2* gene product was used to immunize rabbits. Serum from these rabbits was affinity purified with this same peptide. The resulting antibodies recognize a series of bands on immunoblots of mitochondrial fractions from various yeast strains (Fig. 4). These bands were strongest in mitochondria from strain B-8033, containing the *CYC2* gene on a multicopy plasmid. They were weaker in mitochondria of strain B-7503, containing only the normal chromosomal copy of *CYC2*, and they were absent in strain B-7916, containing a *cyc2* deletion. The major immunologically reactive species ran as a doublet at about 45 kDa. Other, weaker bands that varied with the gene dosage of *CYC2* were visible at 37, 27, and 21 kDa. The reason for discrepancy between the predicted molecular weight of the *CYC2* gene product and the mobilities of the predominant immunologically reactive species on SDS-polyacrylamide gels is not known. It is possible that the protein exists as a covalent multimer, that it contains extensive covalent modifications, or that its intrinsic physical and chemical properties cause anomalous migration during electrophoresis.

Immunoblots of various subcellular fractions from strains B-8033, containing *CYC2* on a multicopy plasmid, and B-7503, containing a single chromosomal copy of *CYC2*, are shown in Fig. 5. When probed with affinity-purified anti-*CYC2* antibodies, nonmitochondrial fractions exhibited a number of bands that appeared to be the result of antibody cross-reactivity, since they were not influenced by *CYC2* gene dosage. In strain B-8033, the major immunologically reactive 45-kDa band can be seen in whole-cell lysate (lane 6) but is present at greatly reduced levels, if at all, in the cytoplasmic fraction (lane 7). This band is also barely detectable in whole-cell extracts (lane 1) from strain B-7503 containing only a single copy of *CYC2*. The *CYC2* gene product is concentrated in mitochondrial fractions from both

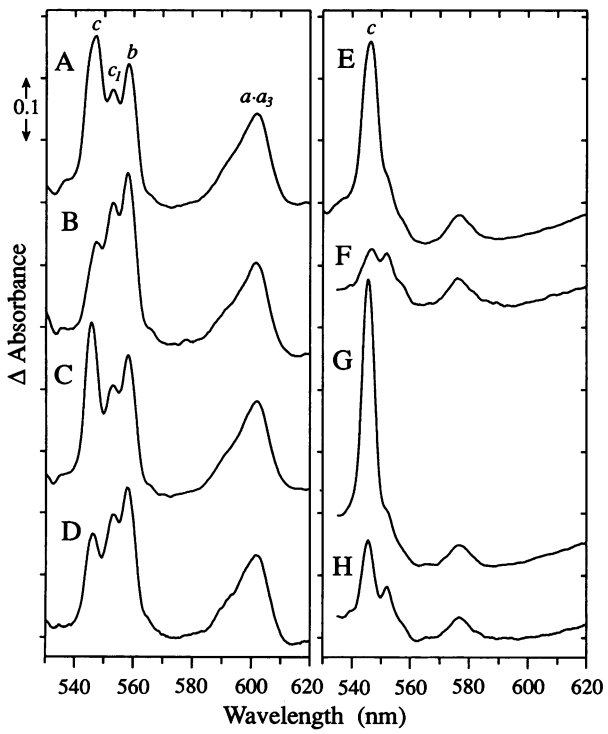

FIG. 3. Low-temperature spectrophotometric recordings of intact cells of the strains listed in Table 1. The α-peaks of cytochromes aa_3, b, c_1, and c are located, respectively, at 602.7, 558.5, 558.3, and 547.3 nm. The *rho*$^-$ strains lack cytochromes aa_3 and b and also contain diminished levels of cytochrome c_1. They also contain some zinc protoporphyrin absorbing at 575 nm. Because of an abnormal promoter, *CYC7-H3* encodes high levels of normal iso-2-cytochrome *c*. Comparison of the different scans demonstrate that *cyc2*$^-$ mutations diminish iso-1-cytochrome *c* (*CYC1*$^+$) more than iso-2-cytochrome *c* (*CYC7-H3*) and that the effects of *cyc2* mutations are enhanced in *rho*$^-$ strains.

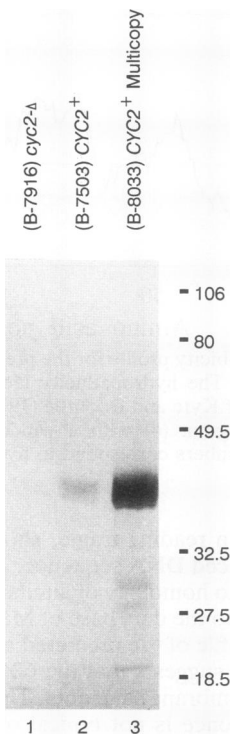

FIG. 4. Antipeptide antibodies recognize the *CYC2* gene product on immunoblots. A total of 30 μg of mitochondrial protein was loaded on each lane of an SDS–12% polyacrylamide gel (20). The gel was transferred to nitrocellulose and probed with antibodies as described in Materials and Methods. Mitochondria were isolated from the following strains: lane 1, B-7916 (cyc2-Δ::*LEU2*$^+$); lane 2, B-7503 (*CYC2*$^+$); lane 3, B-8033 (*CYC2*$^+$ [pBC58]). Numbers to the right of the panels indicate the mobilities of molecular mass standards in kilodaltons.

strains (lanes 3 and 8) and is resistant to proteinase K added externally to mitochondria (lanes 4 and 9), unless detergent is added to disrupt the membranes (lane 5). The overexpressed *CYC2* gene product, however, cannot be completely digested by proteinase K in the presence of detergent (lane 10). This could reflect aggregation of the protein when it is present at higher-than-normal concentrations.

On the basis of results of immunoblotting studies with antibodies recognizing glucose-6-phosphate dehydrogenase, contaminating cytoplasmic proteins represent about 10% of total protein in the mitochondrial fractions presented in Fig. 5. Since the *CYC2* gene product is not a major component of the cytoplasmic fraction and since the amount of total protein in the mitochondrial fractions in Fig. 5 is only 1/10 of the amount of total protein in the cytoplasmic fraction, the band corresponding to *CYC2* protein in the mitochondrial fractions cannot represent cytoplasmic contamination. When assayed by immunoblotting of these same mitochondrial fractions, the marker proteins cytochrome *c* heme lyase, an inner membrane-associated protein exposed to the intermembrane space (7), and cytochrome b_2, a soluble marker of the intermembrane space (3), exhibited subcellular distributions and extents of protease resistance that were similar to those seen for *CYC2* (Fig. 5, lower panels). In addition, we have found enrichment of immunologically detectable *CYC2* protein in a mitochondrial membrane fraction purified by equilibrium density gradient ultracentrifugation (results not shown). Thus, it is unlikely that the *CYC2* gene product is actually a cytoplasmic protein that artifactually cofractionates with mitochondria because it forms protease-resistant aggregates that pellet with mitochondria during subcellular fractionation.

The *CYC2* gene product is involved in mitochondrial import of cytochrome *c*. The subcellular distribution of cytochrome *c* was compared in pairs of *rho*$^+$ and *rho*$^-$ strains that are isogenic except for the replacement of the *CYC2* gene by *LEU2*. All these strains contain deletions of the *CYC1* gene, encoding iso-1-cytochrome *c*; however, they produce high levels of iso-2-cytochrome *c* expressed from the *CYC7-H3* allele. It was necessary to perform the subcellular fractionation in this background, because iso-1-cytochrome *c*, normally the predominant isozyme of cytochrome *c* in *S. cerevisiae*, is unstable in its apo-form and thus cannot be detected in strains with defects in mitochondrial import. Iso-2-cytochrome *c*, on the other hand, is relatively stable as the apo-protein (10).

In the *rho*$^-$ *CYC2*$^+$ strain B-8140, almost all the immunologically detectable cytochrome *c* is contained in the mitochondria in a protease-resistant compartment (Table 2; Fig. 6A [note that 10 times as much protein was loaded on lanes 1, 2, 6, and 7 as on lanes 3 to 5 and 8 to 10]). Thus, cytochrome *c* appears to be imported with the normal high

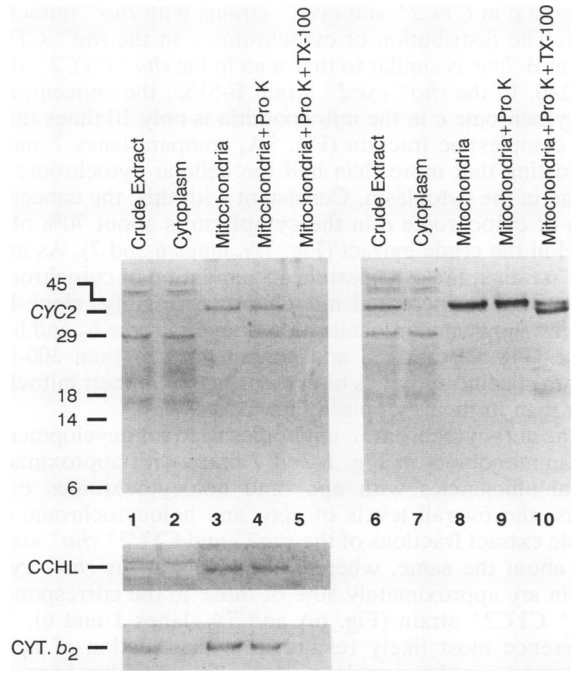

FIG. 5. The *CYC2* gene product is associated with mitochondria. Subcellular fractions were subjected to immunoblotting as described in Materials and Methods. Lanes 1 to 5, subcellular fractions from strain B-7503, containing a normal *CYC2* locus; lanes 6 to 10, Subcellular fractions from strain B-8033, containing the *CYC2* gene on the multicopy plasmid pBC58. Upper panels, the blot was developed with affinity-purified anti-*CYC2* antibody; lower panels, immunoblots of the same subcellular fractions of strain B-7503 as used for lanes 1 to 5 of the upper panel, but developed with anti-cytochrome *c* heme lyase antibodies (middle panel), or anti-cytochrome b_2 antibodies (lower panel). Pro K, samples were treated with proteinase K; TX-100, protease treatment was carried out in the presence of Triton X-100. Numbers to the left of the panels indicate the mobilities of molecular mass standards in kilodaltons. *CYC2* denotes the mobility of the predominant band corresponding to the *CYC2* gene product. CCHL denotes the mobility of cytochrome *c* heme lyase. CYT. b_2 denotes the mobility of cytochrome b_2. For the top two panels, a total of 23 μg of protein (each) was loaded on lanes 1, 2, 6, and 7. A total of 2.3 μg (each) was loaded on lanes 3 to 5 and 8 to 10. The gels shown in these panels contained 12% acrylamide (20). For the bottom panel, 12 μg of protein (each) was loaded on lanes 1 and 2, and 1.2 μg (each) was loaded on lanes 3 to 5. The gel for the bottom panel contained 10% acrylamide (20). The affinity-purified antibody used to detect the *CYC2* protein on this blot was from a different rabbit than that used for Fig. 4. The antibodies from this second animal showed lower overall reactivity than those from the first but recognized fewer background bands in the cytoplasm-containing fractions.

efficiency into *rho*$^-$ *CYC2*$^+$ mitochondria, consistent with previous demonstrations that there are normal levels of holo-iso-2-cytochrome *c* in *rho*$^-$ *CYC2*$^+$ strains (23). Despite repeated attempts with high concentrations of proteinase K, we have been unable to digest all of the mitochondrial holocytochrome *c* with proteinase K in the presence of Triton X-100, as seen in lanes 5 of Fig. 6A and 7A. The high level of holocytochrome *c* in the *CYC2*$^+$ strains, measured by low-temperature spectroscopic examination of whole cells, when considered with the fact that cytochrome *c* heme lyase is located inside mitochondria (7), provides independent evidence that the cytochrome *c* in this *CYC2*$^+$ strain is in fact mitochondrial.

TABLE 2. Relative amounts of immunologically detected cytochrome *c* in subcellular fractions of *cyc2*$^-$ and *CYC2*$^+$ yeast strains

Strain	Concn ratio[a]		% Cytochrome *c* in the cytoplasm[b]
	Mitochondria/ cytoplasm	Crude extract/ cytoplasm	
B-8140 (*rho*$^-$ *CYC2*$^+$)	300	10	10
B-8142 (*rho*$^-$ *cyc2*$^-$)	5	1	80
B-7908 (*rho*$^+$ *CYC2*$^+$)	300	10	10
B-8133 (*rho*$^+$ *cyc2*$^-$)	10	1.5	70

[a] Concentration ratios refer to the ratios of the concentrations of immunoreactive cytochrome *c* per milligram of total protein in the indicated subcellular fractions. The indicated values were measured by comparison of band intensities on immunoblots of dilutions of subcellular fractions containing known amounts of total protein, as described in Materials and Methods.

[b] Cytochrome *c* contents of mitochondria, expressed (to the nearest 10%) as percentages of the total amounts of cellular cytochrome *c*, were calculated from the mitochondria/cytoplasm concentration ratios by assuming that mitochondria constitute 5% of total cellular protein.

Most of the immunologically detectable cytochrome *c* in the *rho*$^-$ *cyc2*$^-$ strain B-8142 is retained in the cytoplasm (Table 2 and Fig. 6A, lane 7), resulting in a concentration of cytoplasmic cytochrome *c* that is approximately 10 times that in B-8140, the isogenic *rho*$^-$ *CYC2*$^+$ strain (compare lanes 2 and 7 of Fig. 6A). The concentration of cytochrome *c* in the mitochondria of the *cyc2*$^-$ strain is approximately five times higher than in the cytoplasm of the same strain

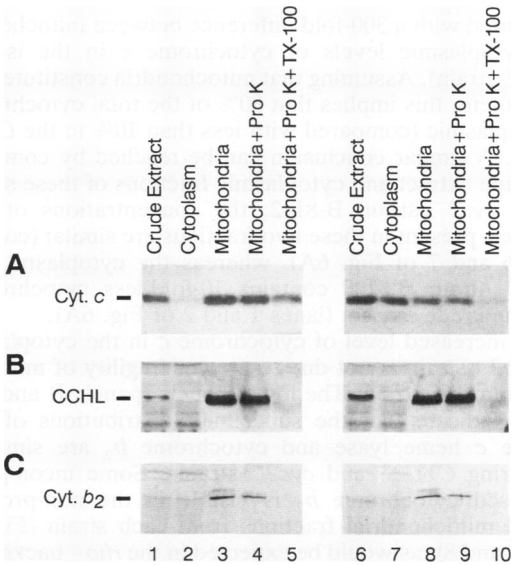

FIG. 6. Apocytochrome *c* accumulates in the cytoplasm of *rho*$^-$ *cyc2*$^-$ cells. Subcellular fractionation and immunoblotting with anti-cytochrome *c*, anti-heme lyase, and anti-cytochrome b_2 antibodies were performed as described in Materials and Methods. Lanes 1 to 5, subcellular fractions of strain B-8140 (*rho*$^-$ *CYC2*$^+$); lanes 6 to 10, subcellular fractions of strain B-8142 (*rho*$^-$ *cyc2*-Δ::*LEU2*$^+$). A total of 20 μg of protein (each) was loaded on lanes 1, 2, 6, and 7, and 2 μg (each) was loaded on lanes 3 to 5 and 8 to 10. (B and C) A total of 40 μg of protein (each) was loaded on lanes 1, 2, 6, and 7, and 4 μg (each) was loaded on lanes 3 to 5 and 8 to 10. The gels for panels A and B contained 10% acrylamide and were prepared and run as described by Schagger and von Jagow (35). The gel for panel C contained 10% acrylamide (20).

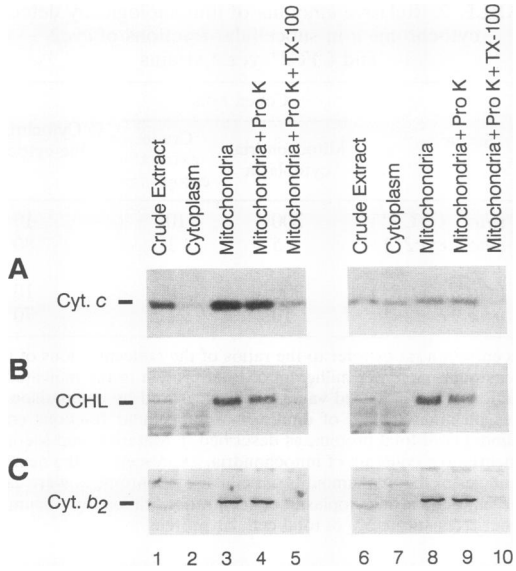

FIG. 7. Apocytochrome *c* accumulates in the cytoplasm of *rho*$^+$ *cyc2*$^-$ cells. Subcellular fractionation and immunoblotting with anti-cytochrome *c* antibodies were performed as described in the legend to Fig. 6. Lanes 1 to 5, subcellular fractions of strain B-7908 (*rho*$^+$ *CYC2*$^+$); lanes 6 to 10, subcellular fractions of strain B-8133 (*rho*$^+$ *cyc2*-Δ::*LEU2*$^+$). (A) A total of 10 μg of protein (each) was loaded on lanes 1, 2, 6, and 7, and 1 μg (each) was loaded on lanes 3 to 5 and 8 to 10. (B and C) A total of 40 μg of protein (each) was loaded on lanes 1, 2, 6, and 7, and 4 μg (each) was loaded on lanes 3 to 5 and 8 to 10.

(compared with a 300-fold difference between mitochondrial and cytoplasmic levels of cytochrome *c* in the isogenic *CYC2*$^+$ strain). Assuming that mitochondria constitute 5% of cell protein, this implies that 80% of the total cytochrome *c* is cytoplasmic (compared with less than 10% in the *CYC2*$^+$ strain). A similar conclusion can be reached by comparing the crude extract and cytoplasmic fractions of these strains. In the *cyc2*$^-$ strain B-8142, the concentrations of cytochrome *c* present in these two fractions are similar (compare lanes 6 and 7 of Fig. 6A), whereas the cytoplasm of the *CYC2*$^+$ strain B-8140 contains 10-fold-less cytochrome *c* than the crude extract (lanes 1 and 2 of Fig. 6A).

The increased level of cytochrome *c* in the cytoplasm of the *cyc2*$^-$ strain is not due to greater fragility of mitochondria from this strain. The lower panels (panels B and C) of Fig. 6 indicate that the subcellular distributions of cytochrome *c* heme lyase and cytochrome b_2 are similar in comparing *CYC2*$^+$ and *cyc2*$^-$ strains. Some incompletely processed cytochrome b_2 is visible in the non-protease-treated mitochondrial fractions from each strain (Fig. 6C, lanes 3 and 8), as would be expected in the *rho*$^-$ background (30). Furthermore, the activity of cytochrome b_2 per milligram of protein, measured in the presence of detergent (7), was at least 150 times greater in the mitochondria than in the cytoplasmic fractions for both B-8140 and B-8142. Thus, reduced levels of mitochondrial holocytochrome *c* in the *cyc2*$^-$ strain, coupled with the increased concentration of cytoplasmic apocytochrome *c*, suggests that the *CYC2* gene product affects mitochondrial import of cytochrome *c*.

Although they are more pronounced in *rho*$^-$ strains, the effects of *cyc2*$^-$ mutations are not confined to *rho*$^-$ backgrounds. Figure 7 and Table 2 show a comparison of the subcellular distributions of immunologically detectable cytochrome *c* in *CYC2*$^+$ and *cyc2*$^-$ strains with *rho*$^+$ mitochondria. The distribution of cytochrome *c* in the *rho*$^+$ *CYC2*$^+$ strain B-7908 is similar to that seen in the *rho*$^-$ *CYC2*$^+$ strain B-8140. In the *rho*$^+$ *cyc2*$^-$ strain B-8133, the concentration of cytochrome *c* in the mitochondria is only 10 times that in the cytoplasmic fraction (Fig. 7A, compare lanes 7 and 9), indicating that more than half the cellular cytochrome *c* is found in the cytoplasm. Consistent with this, the concentration of cytochrome *c* in the cytoplasm is about 70% of the level in the crude extract (Fig. 7A, lanes 6 and 7). As in the *rho*$^-$ strains, the cytoplasmic accumulation of cytochrome *c* is not due to increased mitochondrial fragility, since both strains show similar localization of cytochrome b_2 and heme lyase (Fig. 7B and C) and contain greater than 300-fold-higher specific activities of cytochrome b_2 in their mitochondria than in their cytoplasmic fractions.

The anti-cytochrome *c* antibodies used for development of the immunoblots in Fig. 6 and 7 react with approximately equal efficiencies with apo- and holocytochromes *c* (7). Thus, the overall levels of apo- and holocytochrome *c* in crude extract fractions of the *cyc2*$^-$ and *CYC2*$^+$ *rho*$^-$ strains are about the same, whereas the levels in the *rho*$^+$ *cyc2*$^-$ strain are approximately 50% of those in the corresponding *rho*$^+$ *CYC2*$^+$ strain (Fig. 6A and 7A, lanes 1 and 6). This difference most likely results from degradation of apocytochrome *c* in the cytoplasm. A similar effect has been seen with mutations in the gene encoding cytochrome *c* heme lyase, which have been demonstrated to have no effect on transcription or translation of the genes encoding cytochrome *c* (7). Loss of cytoplasmic apocytochrome *c* could also be occurring during subcellular fractionation. A faint band in the crude extract and cytoplasmic fractions of the *rho*$^-$ *cyc2*$^-$ strain (Fig. 6A, lanes 6 and 7) that is smaller than cytochrome *c* and is recognized by anti-cytochrome *c* antibodies may represent a proteolytic fragment of the cytoplasmic apo-protein, since it is not observed in the *rho*$^-$ *CYC2*$^+$ strain in which the cytochrome *c* is predominantly mitochondrial. Instability of cytoplasmic apocytochrome *c* would mean that the concentrations of this protein that we measure in the cytoplasm actually represent an underestimate of the true effect of *cyc2* mutations.

The cytochrome *c* concentrations calculated from immunoblots of subcellular fractions presented in Table 2 correspond approximately with those derived from low-temperature spectroscopy, shown in Table 1. Differences in the exact values, in the cases of the *cyc2*$^-$ strains, may be attributable to differences in growth conditions between the two experiments, to differences in the standardization for total protein in the two techniques, or to breakdown of the assumption that mitochondria make up 5% of cell protein in all strains.

DISCUSSION

Cytochrome *c* is imported into the mitochondrial intermembrane space via a pathway that appears to be distinct from that followed by many nuclear-encoded mitochondrial proteins (12, 15). In contrast to these other imported proteins, cytochrome *c* does not contain an amino-terminal cleaved targeting signal and its import does not depend on the presence of ATP or an electrical potential across the inner mitochondrial membrane. Furthermore, an excess of apocytochrome *c* cannot competitively inhibit import of other mitochondrial precursors (47). Unlike cytochrome c_1 and cytochrome b_2, which are also targeted to the intermembrane space, import of cytochrome *c* does not appear to

involve targeting to the matrix or inner membrane followed by reexport. Thus, the cellular components involved in cytochrome *c* import may be at least partially distinct from those involved in import of other mitochondrial proteins, consistent with the isolation of mutations that affect transport of cytochrome *c* but not other mitochondrial proteins. It has recently been demonstrated that the import pathways of the intermembrane space proteins cytochrome *c* heme lyase (21) and adenylate kinase (22) differ in important respects from the pathway followed cytochromes b_2 and c_1. Thus, cytochrome *c* may not be the only protein that uses an alternate pathway.

Aside from the structural genes encoding the two isozymes of cytochrome *c* in *S. cerevisiae*, two additional genes have been shown to be capable of strongly and specifically affecting cellular levels of cytochrome *c*. One of these, *CYC3*, encodes cytochrome *c* heme lyase (8, 9). Several recent studies suggest that cytochrome *c* is imported via an initial reversible translocation across the outer mitochondrial membrane followed by trapping of translocated precursor in the intermembrane space (7, 14). This trapping appears to be mediated by the heme lyase. Apocytochrome *c* adopts an unfolded conformation, even in the absence of denaturants (11, 45). Attachment of heme, catalyzed by heme lyase, leads to folding of the precursor, presumably preventing release from mitochondria. Heme lyase does not appear to play a direct role in transport into mitochondria, since it is predominantly located on the inner mitochondrial membrane (7).

We report here the cloning of the *CYC2* gene, a second locus that is capable of decreasing levels of holocytochrome *c* in yeast cells. Accumulation of apocytochrome *c* in certain *cyc2*$^-$ holocytochrome *c*-deficient yeast strains had previously been presented as evidence that the *CYC2* gene acts at a posttranslational step in cytochrome *c* biosynthesis (23). We have detected decreased accumulation of immunologically detectable cytochrome *c* in the mitochondria of certain *cyc2*$^-$ strains, accompanied by substantially increased levels of apocytochrome *c* in the cytoplasm. Thus, *cyc2*$^-$ mutations appear to affect holocytochrome *c* levels by reducing the efficiency of mitochondrial import or accumulation of cytochrome *c*.

The *cyc2* deletion does not appear to affect levels of mitochondrial cytochrome *c* heme lyase or cytochrome b_2, as determined by immunoblotting (Fig. 6B and C and 7B and C). In addition, low-temperature spectroscopy of whole yeast cells demonstrates that *cyc2* mutations do not affect levels of other mitochondrial cytochromes including mitochondrially encoded cytochrome *b*, nuclear-encoded cytochrome c_1, and cytochrome aa_3, which consists of both nuclear- and mitochondrially encoded subunits (31) (Fig. 3). Thus, the effects of the mutation appear to be specific for mitochondrial import of cytochrome *c*.

Although the *CYC2* gene product appears to be involved in mitochondrial import, it is not an essential component of the import apparatus. In strains from which the *CYC2* gene has been deleted, there is never less than about 10% of the normal level of holocytochrome *c*. This observation is consistent with either of two possibilities for the role of the *CYC2* gene product: (i) The *CYC2* protein serves to increase the efficiency or rate of mitochondrial import or accumulation without being absolutely necessary for either of these processes, and (ii) the *CYC2* protein plays a critical role in import or accumulation, but one or more additional genes present in the yeast genome are capable of substituting for *CYC2*. If mutations in such additional genes were pleiotropic or lethal or could only be detected in the absence of a functional *CYC2* gene product, they could have been missed in previous intensive searches for mutations affecting cytochrome *c* levels (5, 36, 41, 42). Possible dispensable roles for the *CYC2* protein could include binding of precursor at the mitochondrial surface, alteration of the assembly or localization of a transporter at the outer mitochondrial membrane, or alteration of the localization or activity of heme lyase. However, the absence of any effect of *cyc2* mutations on the subcellular localization of heme lyase presented in Fig. 6B and 7B provides evidence against this last possibility. The presence of one or two potential transmembrane segments in the hydrophobicity profile of the predicted amino acid sequence of the *CYC2* gene product is consistent with possible roles in membrane anchoring or transport.

Differential effects of *cyc2* mutations on iso-1- and iso-2-cytochromes *c*. *cyc2* mutations affect cellular levels of holo-iso-1-cytochrome *c* more strongly than they do levels of iso-2-cytochrome *c* (Table 1). Assuming that import of each isozyme of cytochrome *c* is affected by *cyc2* mutations in the same way, this difference is consistent with different susceptibilities of the apo-forms of the two isozymes to degradation. When retained in the cytoplasm, apo-iso-2-cytochrome *c* has a much longer half-life than apo-iso-1-cytochrome *c* (10). Thus, in the presence of a partial defect of import into mitochondria, as in *cyc2* mutants, apo-iso-2-cytochrome *c* may accumulate to high levels in the cytoplasm, eventually driving moderate levels of import and subsequent heme attachment. The same defect in import would, on the other hand, lead to rapid degradation of apo-iso-1-cytochrome *c*, precluding significant cytoplasmic accumulation of the precursor and subsequent import into mitochondria.

We have not detected increased apo-iso-1-cytochrome *c* in the cytoplasm of *cyc2*$^-$ strains and would not expect to be able to because of the lability of this isozyme. Thus, the idea that the *cyc2* mutation affects import of both isozymes of cytochrome *c* is based on the similarity of the behaviors of the two isozymes in in vitro import assays, in which degradation is not a major factor (9). An effect of *cyc2* mutations specifically on mitochondrial import of iso-1-cytochrome *c* has yet to be rigorously demonstrated.

***rho*$^-$ effect in *cyc2* mutations.** By definition, *rho*$^-$ strains are completely deficient in mitochondrially encoded proteins, such as cytochrome *b* and the subunits I to III of cytochrome oxidase. However, nuclear-encoded mitochondrial proteins, which constitute at least 80% of mitochondrial proteins, also can be partially deficient in *rho*$^-$ strains. For example, in *rho*$^-$ strains, the nuclear-encoded protein cytochrome c_1 is generally present at reduced levels, compared with those seen in normal *rho*$^+$ strains (37). Furthermore, mutationally altered forms of certain nuclear components may be greatly reduced in *rho*$^-$ strains compared to with *rho*$^+$ strains. In particular, certain mutationally altered δ-aminolevulinic acid synthetase (*hem1*) (34) and iso-2-cytochrome *c* (*cyc7*) (6) proteins appear at near normal levels in *rho*$^+$ strains and are completely or almost completely deficient in *rho*$^-$ strains. In contrast, normal *HEM1*$^+$ and *CYC7*$^+$ gene products are present at the same level in *rho*$^+$ and *rho*$^-$ strains. Although the mechanism of the diminution of normal and mutant components in *rho*$^-$ strains is unknown, the lack of proper assembly because of primary *rho*$^-$ deficiencies may cause degradation, as has been suggested for incomplete assembly of coenzyme QH_2-cytochrome *c* reductase subunits in *rho*$^+$ mutants (2). The lower holocytochrome *c* content in *rho*$^-$ *cyc2*$^-$ strains may be due to degradation of a hypothetical component that acts in parallel

with *CYC2* as part of the mitochondrial apparatus involved in cytochrome *c* maturation or import.

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