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Overlapping Specificities of the Mitochondrial Cytochrome *c* and c_1 Heme Lyases*[S]

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Heme attachment to the apoforms of fungal mitochondrial cytochrome *c* and c_1 requires the activity of cytochrome *c* and c_1 heme lyases (CCHL and CC_1HL), which are enzymes with distinct substrate specificity. However, the presence of a single heme lyase in higher eukaryotes is suggestive of broader substrate specificity. Here, we demonstrate that yeast CCHL is active toward the non-cognate substrate apocytochrome c_1, *i.e.* CCHL promotes low levels of apocytochrome c_1 conversion to its holoform in the absence of CC_1HL. Moreover, that the single human heme lyase also displays a broader cytochrome specificity is evident from its ability to substitute for both yeast CCHL and CC_1HL. Multicopy and genetic suppressors of the absence of CC_1HL were isolated and their analysis revealed that the activity of CCHL toward cytochrome c_1 can be enhanced by: 1) reducing the abundance of the cognate substrate apocytochrome *c*, 2) increasing the accumulation of CCHL, 3) modifying the substrate-enzyme interaction through point mutations in CCHL or cytochrome c_1, or 4) overexpressing Cyc2p, a protein known previously only as a mitochondrial biogenesis factor. Based on the functional interaction of Cyc2p with CCHL and the presence of a putative FAD-binding site in the protein, we hypothesize that Cyc2p controls the redox chemistry of the heme lyase reaction.

The *c*-type cytochromes are a widespread class of essential metalloproteins that are located on the positive side of energy-transducing membrane systems. They function in electron transfer (1) but are also involved in the cellular death pathways in vertebrates by signaling mitochondrial status (2). Their distinctive feature is the covalent attachment of the prosthetic group via thioether linkages between the vinyl chains of heme and the cysteines of the C*XX*CH motif on the apocytochrome. If cytochromes *c* are among the best characterized molecules at the structural level (3, 4), the biochemistry of their maturation is poorly understood and needs to be deciphered. Key questions in defining the steps and the biochemical requirement to complete holocytochrome *c* maturation have been addressed and the general conclusion is that heme and apocytochromes need to be transported to the positive side of the energy-tranducing membrane and maintained in a reduced form prior to catalysis of heme attachment, the terminal step of the maturation process (1, 5–9). Remarkably, three distinct assembly pathways (systems I, II, and III) have been recognized through genetic analysis of *c*-type cytochrome maturation in prokaryotic and eukaryotic organisms (for review see Refs. 1, 3, and 8–12). Experimental investigations in bacterial and plastid models for systems I and II led to the view that cytochrome *c* maturation is a complex process that requires the activity of numerous gene products (1, 11–16). Common to systems I and II is the operation of a cytochrome *c* assembly machinery with heme delivery/handling systems (8, 17–21) and multiple redox components dedicated to the maintenance of co-factor and cysteine sulhydryls in a reduced state prior to the heme lyase reaction (9, 15, 22–26).

System III was discovered through studies in *Neurospora crassa* and *Saccharomyces cerevisiae* and seems to be restricted to mitochondria of vertebrates, invertebrates, apicomplexan parasites, and green algae as gauged from genome analysis (see Fig. 1 of Supplemental Materials and Ref. 8). In contrast to systems I and II, system III appears surprisingly less complex as extensive genetic screens led to the discovery of only two assembly factors, the cytochrome *c* heme lyase (CCHL)[1] and the cytochrome c_1 heme lyase (CC_1HL), for the two *c*-type cytochromes found in the mitochondrial intermembrane space, cytochrome *c* and cytochrome c_1, respectively (27–30). It is conceivable that other assembly factors have escaped genetic identification because of redundancy of function or participation in essential mitochondrial processes.

The specificity of each lyase for its respective substrate was established through genetics in the yeast system (28, 31, 32) but needs to be re-examined in the context of the situation where genomes of multicellular eukaryotes, including human, reveal one heme lyase (referred to as HCCS) for both cytochrome *c* and cytochrome c_1 (see Fig. 1 of Supplemental Materials). The relevance of the study of the system III assembly pathway to human health has now become obvious with the finding that loss of CCHL causes a neurodevelopmental disorder with cardiomyopathic manifestations (33). The CCHL and CC_1HL are related in sequence (35% amino acid identity) and display, with the notable exception of *Plasmodium falciparum* and *Chlamydomonas reinhardtii* CC_1HL, one to four typical heme regulatory motifs (see Fig. 1 of Supplemental Materials)

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[1] The abbreviations used are: CCHL, cytochrome *c* heme lyase; CC_1HL, cytochrome c_1 heme lyase; HCCS, holocytochrome *c* synthetase; LDS, lithium dodecyl sulfate; ORF, open reading frame; BLAST, basic local alignment search tool; DTT, dithiothreitol.

TABLE I
Genotypes and sources of yeast strains

Strain	Genotype	Source
W303–1A	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1*	R. Rothstein[a]
W303–1B	*α ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1*	R. Rothstein[a]
SMY4	*α ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyc3::loxP-kan-loxP*	This study
SMY1	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyt2::his5*$^+$	This study
YCT1–11A	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyt2::his5*$^+$	This study[b]
UV9	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 CYC3–9 cyt2::his5*$^+$	This study
UV14	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 CYT1–14 cyt2::his5*$^+$	This study
UV17,48	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 CYT1–48 cyt2::his5*$^+$	This study
UV16,18,34,43,50	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 CYT1–34 cyt2::his5*$^+$	This study
R2	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyt2::his5*$^+$ *su2*	This study
R19	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyt2::his5*$^+$ *su19*	This study
CC1–2D (CC1–5A)	*α ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyc3::loxP-kan-loxP cyt2::his5*$^+$	This study[c]
YPH1	*a ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyc2::hph*	This study
PHT3	*α ade2–1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyt1::LEU2*	Ref. 53
YPH9–2A	*a leu2–3,112 his3–11,15 ura3–1 cyc1::kan*	This study[d]
W303ΔCYC7	*α ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 cyc7Δ::TRP1*	A. Tzagoloff
JNY33	*α ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 imp2::LEU2*	Ref. 68

[a] Department of Human Genetics, Columbia University.
[b] YCT1–11A is a spore from the cross between SMY1 and YPH1.
[c] CC1–2D (α) and CC1–5A (a) are spores from the cross between SMY1 and SMY4.
[d] YPH9–2A is a spore from the cross between YPH1 and a strain carrying a null-allele (*cyc1::kan*) in the *CYC1* gene.

that are present also in several other heme-binding proteins such as the transcription factor HapI (34) and heme oxygenase (35). The interaction of CCHLs with heme via the heme regulatory motif (36) and apocytochrome (37, 38) led to the proposal that these assembly factors participate in the biogenesis of cytochromes *c* by catalyzing thioether bond formation. Despite the considerable body of work from the groups of Neupert and Sherman on the mechanism of the heme lyase reaction, including the *in organello* reconstitution of heme linkage to apocytochrome *c* (reviewed in Refs. 39 and 40), the function of the CCHLs has not been thoroughly detailed. Ultimate biochemical proof of their function awaits a direct enzymatic assay of cytochrome heme lyase activity of the purified proteins (28, 41, 42). It is also not known whether other accessory factors assist the heme lyases in the formation of holocytochromes *c*. One such factor was suspected to be Cyc2p, a mitochondrial inner membrane protein identified through a genetic screen for mutants deficient in holocytochrome *c*. The function of Cyc2p in the CCHL pathway remains undeciphered (31, 43). However, on the basis that *cyc2* mutations display a pleiotropic phenotype, it was concluded that Cyc2p controls some general aspect of mitochondrial biogenesis and influences holocytochrome *c* synthesis only indirectly (44, 45).

In this paper, we have addressed the question of heme lyase specificities in yeast. We show that whereas CC_1HL is only active in the assembly of cytochrome c_1, CCHL in addition to its well established function in the biogenesis of holocytochrome *c* is also able to participate in the conversion of apocytochrome c_1 to its holoform. We also demonstrate by functional complementation of the yeast *cchl* and *cc*$_1$*hl* mutants that the single heme lyase in human and mouse exhibits both the CCHL and CC_1HL activities. Through multicopy and genetic suppressor analyses, we revealed different mechanisms by which CCHL specificity is modified in favor of its non-cognate substrate apocytochrome c_1. We also provide evidence that Cyc2p interacts functionally with CCHL in the assembly of holocytochrome c_1 and have identified a putative FAD-binding site in a domain predicted to be exposed to the mitochondrial intermembrane space. The cytochrome specificity of the heme lyases and the putative function of Cyc2p in the CCHL pathway are discussed.

EXPERIMENTAL PROCEDURES

Construction, Manipulation, and Growth of Yeast Strains—All the yeast strains used in the course of this study are listed in Table I. *S. cerevisiae* wild type strains W303-1A and W303-1B are labeled WT in the figures. Strains carrying null alleles in the *CYC1*, *CYC2*, *CYC3*, *CYC7*, *CYT1*, *CYT2*, or *IMP2* genes are, respectively, labeled Δ*cyc1*, Δ*cyc2*, Δ*cchl*, Δ*cyc7*, Δ*cyt1*, Δ*cc*$_1$*hl*, or Δ*imp2* in the figures. The suppressed strains obtained spontaneously (Rx) or after UV-induced mutagenesis (UVx) are designated Δ*cc*$_1$*hl sux* when the suppressor allele is recessive and Δ*cc*$_1$*hl SUx* when the suppressor allele is dominant. Null alleles of yeast genes in the W303 background were constructed using a PCR-based method. The *hphMX4* (46), *loxP-kanMX-loxP* (47), and *HIS3MX6* (48) modules were chosen to inactivate the wild type copy of the *CYC2*, *CYC3*, and *CYT2* genes, respectively. Cells were transformed by the lithium acetate procedure of Schiestl and Gietz (49) or the one-step technique (50). The multicopy wild type genomic library constructed in the *URA3*-based pFL44L vector (a generous gift from F. Lacroute) was used to search for suppressor genes able to alleviate the respiratory deficiency of the Δ*cchl* and Δ*cc*$_1$*hl* mutants. Transformants were plated on medium lacking uracil and replicated on glycerol medium to select for respiratory proficiency. Plasmids retrieved through the multicopy suppressor screen were extracted from yeast transformants and propagated in *Escherichia coli* strains (51). Media used for *S. cerevisiae* have been described elsewhere (52–54). Glucose or galactose were used as fermentable substrates and glycerol, ethanol, ethanol/glycerol, or lactate as respiratory substrates.

Genetic Methods—Spontaneous or UV-induced respiratory-competent revertants were selected from the SMY1 strain (Δ*cc*$_1$*hl*). Spontaneous revertants were isolated from cells grown to stationary phase, which were plated on lactate medium and incubated at 28 °C for 15 days. UV mutagenesis was performed using a UV source (254 nm) placed at a distance of 12 cm from yeast cells. Cells were collected at a late logarithmic phase and plated on glucose medium before mutagenesis. Plates were then irradiated in the dark for 10 s, incubated for 3 days at 28 °C, and replica-plated on glycerol medium. Under these conditions, 38% lethality was observed and $\sim 10^3$ revertants were recovered from 2×10^9 irradiated cells. No spontaneous ($<10^{-10}$) or UV-induced revertants ($<2 \times 10^{-9}$) could be isolated from the SMY4 strain (Δ*cchl*). All the methods employed for the genetic analysis of the suppressor strains have been published elsewhere (52). Molecular identification of the mutations in the mapped suppressor genes was achieved by PCR amplification of the candidate ORF from genomic DNAs of the suppressed strains. Sequencing of the PCR product on both strands confirmed the presence of the suppressor mutation.

Plasmids Construction—DNA manipulations were carried out following published procedures (55). *E. coli* strains XL1-blue, MR32 (a *recA*$^-$ derivative of MC1061, a generous gift from Dr E. Petrochilo) and DH5α were used as hosts for recombinant DNA techniques. The heterologous expression of human and mouse *HCCS* cDNAs was achieved by using the multicopy yeast expression vector pFL61 (56). Human and mouse *HCCS* ORFs were amplified using NotI-engineered oligonucleotides as primers and plasmids harboring the cloned cDNAs as templates (57). NotI-digested PCR products were cloned at the NotI site of pFL61 and recombinant plasmids expressing the human (pFL61-*HCCS Hs*) and mouse HCCS (pFL61-*HCCS Mm*) in the sense orientation with respect to the *PGK* promoter in pFL61 were chosen for this study. The plasmids pFL61-*CCHL Sc* and pFL61-*CC*$_1$*HL Sc* were constructed in a

similar way by cloning the ORFs of the yeast *CYC3* and *CYT2* genes, respectively, in pFL61. The plasmids pFL44L-*CCHL*, pFL44L-*CC$_1$HL*, and pFL44L-*CYC2* were generated as follows: a 1.3-kb SspI-SalI genomic fragment containing the entire CCHL-encoding gene was cut from plasmid pRS316-*CYC3* (a gift from R. Lill) and cloned into the PvuII-SalI sites of pFL44L; the CC$_1$HL encoding gene was cloned into pFL44L as a 1-kb SacI-SphI fragment from plasmid pGEM4-*CYT2* (58). *CYC2* was cloned into pFL44L as a 1.7-kb PCR fragment using primers with engineered SacI and SalI sites and the *CYC2*-harboring plasmid retrieved from the multicopy suppressor screen as a template.

RNA Analysis—RNAs were extracted from cells grown in complete galactose medium (59). 20 μg of total RNAs was prepared as in Chanfreau *et al.* (59) and separated by electrophoresis in a 1.5% agarose denaturing gel. RNAs were immobilized by transfer to nylon membranes (N+ Amersham Biosciences) followed by UV cross-linking. Probes were labeled with [α-^{32}P]dCTP by random primer labeling of PCR amplified DNA using the "Ready-to-go DNA labeling beads" kit (Amersham Biosciences). The abundance of *CYC1* and *CYC7* mRNAs was monitored using a 354-bp PCR product amplified with *CYC1*-specific oligonucleotides (5′-GACTGAATTCAAGGCCGGTTCTG-3′ and 5′-GATATCGACAAAGGAAAAGGGG-3′) and a 355-bp PCR product amplified with *CYC7*-specific oligonucleotides (5′-CAAGAAGATCACATACAAGC-3′ and 5′-GCAAACGCCATCTTGGTACCAGG-3′) as probes. Membranes were pre-hybridized for 30 min in 15 ml of Rapid hybridization buffer (Amersham Biosciences), and hybridized for 1 h at 65 °C (~2.5 × 10^5 cpm/ml). Stringent washes were performed as described in Laz *et al.* (60). Under these conditions, no significant cross-hybridization was observed between the *CYC1* probe and *CYC7* mRNA and between the *CYC7* probe and *CYC1* mRNA. For quantification of transcripts, signals were normalized to that for *RPL18A* mRNA, which encodes the cytosolic ribosomal protein L18A (61).

Protein Preparation and Analysis—Mitochondria were purified from yeast grown in galactose medium as described earlier (62) and the mitochondrial protein concentration was determined using the Bradford reagent (Sigma) or the Coomassie protein assay reagent (Pierce). Soluble cytochrome *c* and membrane-bound cytochrome c_1 were fractionated by sodium carbonate treatment of mitochondrial proteins according to Kermorgant *et al.* (62). Extraction of proteins from whole cells was performed as detailed in Yaffe (63) and the protein concentration was assayed using Folin-Ciocalteu's phenol reagent (Merck). Protein samples were analyzed through LDS-PAGE (4 °C) or SDS-PAGE (room temperature) and subsequently immobilized by electrotransfer to polyvinylidene difluoride or nitrocellulose membranes (0.45 μm). The different intermediates of cytochrome c_1 maturation were resolved by overnight electrophoresis (18 mA) using a 1.5 mm × 14 cm resolving gel. For heme stain of mitochondrial cytochromes *c*, samples were reduced with DTT on ice prior to electrophoresis. Carbonate-treated pellet and supernatant fractions (5 mM final concentration of DTT in the extraction buffer) or DTT-treated mitochondrial proteins (50 mM DTT) were separated by LDS-PAGE (64). The heme-associated peroxidase activity was revealed directly on membrane-transferred *c*-type cytochromes by the enhanced chemiluminescence method from Pierce (Super Signal West Dura or West Pico). Polyclonal antibodies raised against CCHL (R. Lill, University of Marburg, Marburg), cytochrome *c* and Tim54 (C. Koehler, UCLA, Los Angeles), cytochrome c_1 (A. Tzagoloff, Columbia University, New York), and monoclonal antibody against porin (Molecular Probes) were used for immunodetection of immobilized proteins. Bound antibodies were detected by horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies or ^{125}I-protein A, as indicated in the legends.

RESULTS

Conversion of Apo to Holocytochrome c_1 by the Non-cognate Lyase CCHL—To re-investigate the question of the substrate specificities of the heme lyases, we constructed null mutations in the yeast *CCHL*[2] and *CC$_1$HL* genes and analyzed the phenotypes associated with those mutations at the level of respiratory growth and *c*-type cytochromes assembly. Because cytochrome *c* and cytochrome c_1 are both essential for electron transfer, the absence of either heme lyase was predicted to result in complete loss of respiratory growth. Surprisingly, we found that whereas the absence of CCHL leads to a tight respiratory deficiency, the absence of CC$_1$HL does not completely abolish the respiratory growth and results in a leaky phenotype on all the respiratory substrates we tested (Fig. 1 and data not shown). The slow respiratory growth phenotype[3] of the Δ*cc$_1$hl* strain segregated along with the null-allele in a genetic cross and was compensated upon expression of the CC$_1$HL-encoding gene (Fig. 3*A*). It is likely that the low respiratory activity of the Δ*cc$_1$hl* mutant is attributable to the formation of holocytochrome c_1 by the weak activity of the non-cognate CCHL.

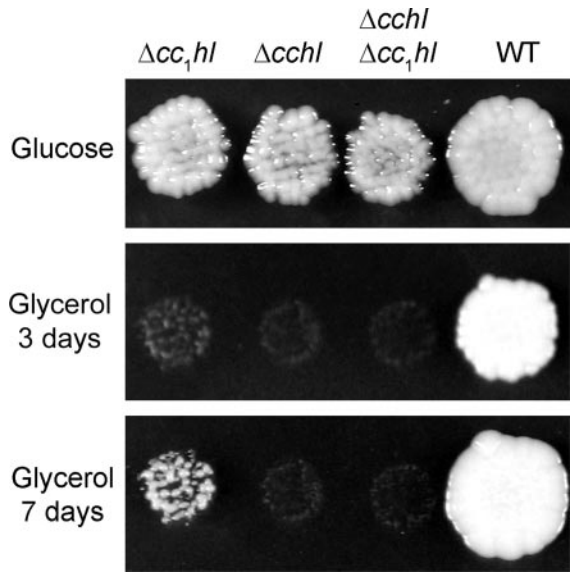

FIG. 1. **Respiratory growth of heme lyase mutants.** WT (W303-1A), Δ*cc$_1$hl* (SYM1), Δ*cchl* (SMY4), and Δ*cchl* Δ*cc$_1$hl* (CC1-5A) strains were grown on fermentable (glucose) or respiratory (glycerol) substrates for 3 or 7 days at 28 °C.

Spectral and heme staining analyses showed that the *cchl*-null strain was deficient only in the assembly of holocytochrome *c* and displays wild type abundance of holocytochrome c_1 (Fig. 2, *A* and *B*), confirming previous work that holocytochrome c_1 is synthesized in a separate pathway (31). No cytochromes aa_3 were spectrally detectable in the Δ*cchl* mutant. This phenotype, however, is not a specific trait of the *cchl*-null mutant but results from loss of cytochrome *c* oxidase assembly as a secondary effect in the absence of holocytochrome *c* (65, 66).

In the Δ*cc$_1$hl* mutant, the abundance of holocytochrome c_1 could not be monitored by whole cell spectral analysis because of the increased level of holocytochrome *c* (see Fig. 2*A* and also Ref. 30) that masked the absorption peak of holocytochrome c_1. Enhanced levels of holocytochrome *c* have already been reported for many nuclear and mitochondrial yeast respiratory deficient mutants (67). A sensitive heme staining procedure on the pellet fraction enabled us to estimate that ~5% of wild type holocytochrome c_1 was synthesized in the *cc$_1$hl* mutant (Fig. 2*C*). The finding that this residual amount of holocytochrome c_1 was no longer observed in the absence of both heme lyases indicates that CCHL displays some activity in the maturation of holocytochrome c_1 (Fig. 2*C*). Immunoblotting analyses using an anti-cytochrome c_1 antibody showed that the intermediate form of apocytochrome c_1, which is the substrate for the heme attachment reaction, accumulated in the Δ*cc$_1$hl* and Δ*cchl* Δ*cc$_1$hl* mutants (data not shown). As a control, we used a null-mutant in the *IMP2* gene that encodes the protease re-

[2] For simplification, the *CYC3* gene specifying CCHL and the *CYT2* gene encoding CC$_1$HL will be referred to as *CCHL* and *CC$_1$HL* genes, respectively, throughout the manuscript.

[3] We also observed a slow respiratory growth phenotype with the YS10 strain carrying a disrupted allele of the *CC$_1$HL* gene (30).

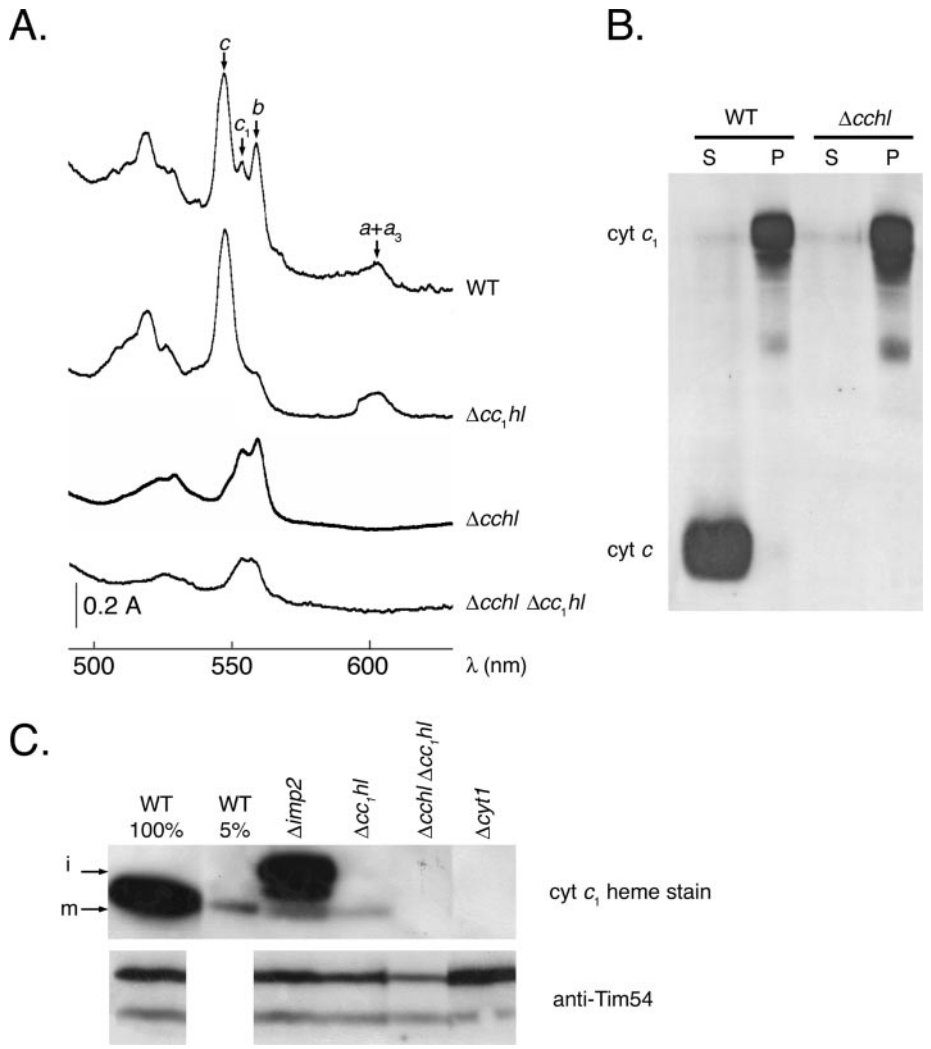

FIG. 2. **Assembly of cytochrome *c* and c_1 in heme lyase mutants.** Strains used were: WT (W303-1A), Δ*cc*$_1$*hl* (SMY1), Δ*cchl* (SMY4), Δ*cchl* Δ*cc*$_1$*hl* (CC1-2D), Δ*cyt1* (PHT3), Δ*imp2* (JNY33). *Panel A*, cytochrome absorption spectra of heme lyase mutants. Low temperature absorption spectra of cells grown in galactose were recorded with a Cary 400 spectrophotometer as described by Claisse *et al.* (81). The *arrows* indicate the absorption maxima of the α bands of cytochromes *c* (546 nm), c_1 (552 nm), *b* (558 nm), and $a + a_3$ (602 nm). Absorption peaks (554 and 557–559 nm) detected in the Δ*cchl* Δ*cc*$_1$*hl* mutant are also present in a Δ*cyt1* Δ*cc*$_1$*hl* strain and correspond to unidentified species, presumably intermediates of the tetrapyrrole pathway. *Panel B*, heme stain of mitochondrial *c*-type cytochromes. 300 μg of mitochondrial protein purified from cells grown on galactose medium was treated with sodium carbonate. Under these conditions, soluble holocytochrome *c* is extracted in the supernatant (*S*), whereas membrane-bound holocytochrome c_1 is found in the pellet (*P*). The supernatant and pellet fractions were separated by electrophoresis in a 12.5% LDS-polyacrylamide gel and transferred to nitrocellulose membrane. Detection of holocytochrome *c* and c_1 was performed by heme staining using the Super Signal West Pico (Pierce). *Panel C*, accumulation of holocytochrome c_1 in heme lyase mutants. 140 μg of mitochondrial protein from cells grown at 28 °C in galactose medium was analyzed for the abundance of holocytochrome c_1. DTT-treated samples were separated in a 12% LDS-polyacrylamide gel at 4 °C. For an estimation of the cytochrome c_1 abundance, a dilution of the wild type sample was loaded on the gel. Following electrophoresis, the gel was transferred to polyvinylidene difluoride membrane before heme staining by chemiluminescence and immunodecoration with antisera against cytochrome c_1 or Tim54 as a loading control. Heme staining was performed using the Super Signal West Dura (Pierce). Bound anti-Tim54 antibodies were detected with ^{125}I-Protein A. Heme staining and autoradiographic exposures were performed under the same conditions for all samples. *i*, intermediate form of holocytochrome c_1; *m*, mature holocytochrome c_1. The minor heme-containing species of cytochrome c_1 that accumulates in the Δ*imp2* mutant is probably not a kinetically competent pathway intermediate and might result from degradation of the i-form. However, the accumulation of some mature holocytochrome c_1 in the absence of Imp2p indicates that i-cytochrome c_1 can be cleaved by an Imp2p-independent processing activity (68).

sponsible for the processing of the intermediate form of cytochrome c_1 to its mature holoform (68). In agreement with the previous finding that heme attachment precedes proteolytic processing by Imp2p (38, 68, 69), the Δ*imp2* mutant accumulated predominantly the heme-containing intermediate form of cytochrome c_1 (Fig. 2*C* and data not shown). Taken together, these results demonstrate that CCHL displays a weak activity toward the intermediate form of apocytochrome c_1 and is able to catalyze its assembly to the mature holoform, whereas CC_1HL activity appears restricted to apocytochrome c_1.

Overexpression of CCHL Compensates for the Absence of CC_1HL—If CCHL can promote heme attachment to its non-cognate substrate apocytochrome c_1, then it might be possible that CC_1HL catalyzes a reaction with apocytochrome *c*, because both heme lyases are in the same compartment (30, 38, 70, 71). To assess cross-catalysis of the heme lyases, we tested whether overexpression of CCHL or CC_1HL from a multicopy expression vector could result in increased activity of each lyase for its non-cognate cytochrome substrate. As shown in Fig. 3*A*, overexpression of CCHL suppresses the respiratory deficiency of the Δ*cc*$_1$*hl* and Δ*cchl* Δ*cc*$_1$*hl* mutants. Doubling times of transformants growing in liquid respiratory medium and expressing CCHL indicated that suppression mediated by CCHL is less effective than complementation by wild type CC_1HL (see Table II). Indeed, the amount of spectrally detected holocytochrome c_1 was not restored to wild type upon expres-

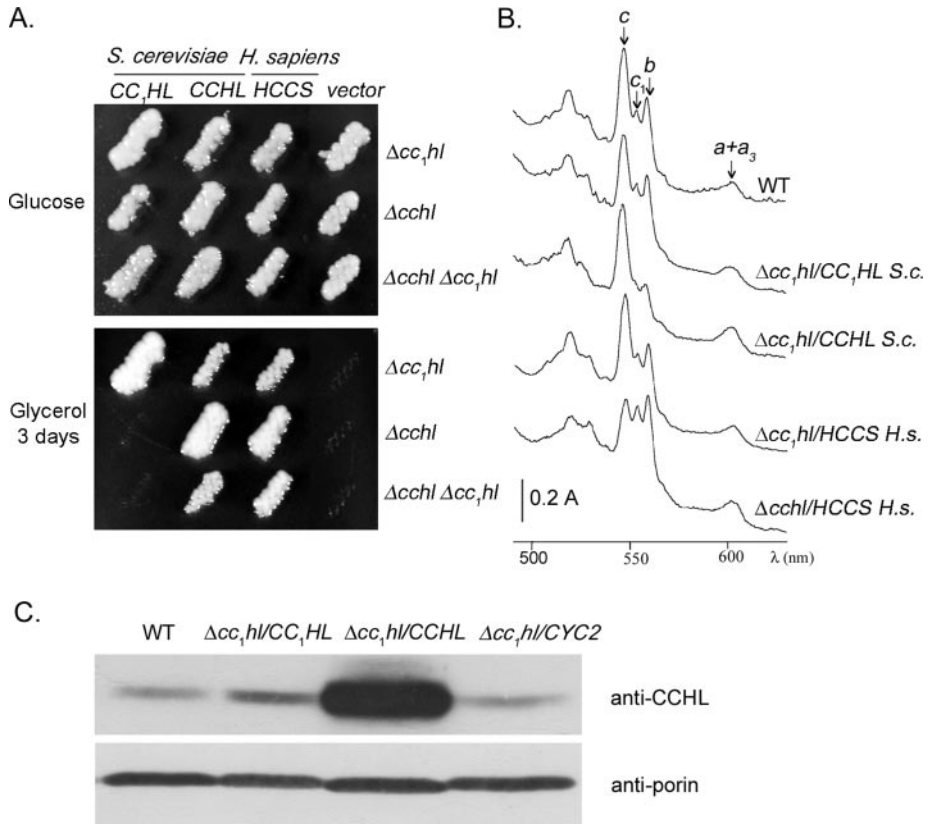

FIG. 3. **Effect of the overexpression of human HCCS, yeast CCHLs or Cyc2p in heme lyase mutants.** Strains used were: WT (W303-1A), Δ*cc*$_1$*hl* (SMY1), Δ*cchl* (SMY4), and Δ*cchl* Δ*cc*$_1$*hl* (CC1-5A). *Panel A*, respiratory growth of the heme lyase mutants expressing yeast CCHLs or human HCCS. Δ*cc*$_1$*hl*, Δ*cchl*, and Δ*cchl* Δ*cc*$_1$*hl* strains were transformed by the multicopy expression vector pFL61 (vector) or pFL61-based plasmids expressing *S. cerevisiae* (*s.c.*) *CC*$_1$*HL*, *CCHL*, or *Homo sapiens* (*H.s.*) *HCCS*. Transformants were selected on minimal glucose medium, replica-plated on glycerol media, and incubated for 3 days at 28 °C. *Panel B*, cytochrome spectra of the heme lyase mutants expressing yeast CCHLs or human HCCS. Low temperature absorption spectra of cells grown in glycerol were recorded with a Cary 400 spectrophotometer as already described (81). The *arrows* indicate the absorption maxima of the α bands of cytochromes *c* (546 nm), c_1 (552 nm), *b* (558 nm), and $a + a_3$ (602 nm). The spectrum of the Δ*cchl*/*CCHL S.c.* transformant is not shown but was found to be identical to that of Δ*cc*$_1$*hl*/*CC*$_1$*HL S.c.* The spectra of the Δ*cc*$_1$*hl*/*CCHL S.c.* and Δ*cchl* Δ*cc*$_1$*hl*/*CCHL S.c.* transformants were also comparable. Similarly, expression of human *HCCS* in the Δ*cchl* and Δ*cchl* Δ*cc*$_1$*hl* results in identical spectra. *Panel C*, abundance of CCHL upon overexpression of *CCHL*, *CC*$_1$*HL*, or *CYC2*-encoding genes in the Δ*cc*$_1$*hl mutant*. WT strain or Δ*cc*$_1$*hl* mutant transformed by multicopy plasmids pFL61-*CC*$_1$*HL*, pFL61-*CCHL*, or pFL44L-*CYC2* were grown at 28 °C in glycerol medium and analyzed for the accumulation of CCHL. Samples corresponding to 160 μg of whole cell protein extracts were separated on a 16% acrylamide, 0.5% bisacrylamide SDS gel and transferred to a nitrocellulose membrane. Immunodecoration was performed with antibodies against CCHL and porin as a loading control. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibody.

TABLE II
Doubling times of yeast strains grown in glycerol medium (28 °C)

Strain	Plasmid	Duplication time
W303–1A		2 h 30
Δcchl	pFL61-*CCHL Sc*	2 h 20
Δ*cc*$_1$*hl*	pFL61-*CC*$_1$*HL Sc*	2 h 20
Δ*cc*$_1$*hl*	pFL61-*CCHL Sc*	6 h 15
Δ*cchl* Δ*cc*$_1$*hl*	pFL61-*CCHL Sc*	7 h 10
Δ*cchl*	pFL61-*HCCS Hs*	2 h 20
Δ*cc*$_1$*hl*	pFL61-*HCCS Hs*	2 h 45
Δ*cchl* Δ*cc*$_1$*hl*	pFL61-*HCCS Hs*	2 h 45

sion of CCHL in the Δ*cc*$_1$*hl* and Δ*cchl* Δ*cc*$_1$*hl* strains (Fig. 3*B* and data not shown). This partial restoration of holocytochrome c_1 could account for the weak suppression of the respiratory growth deficiency and suggests that the abundance of cytochrome c_1 is limiting for respiratory activity. We verified by immunoblot analysis with an anti-CCHL antibody that overexpression of the CCHL-encoding gene in the *cc*$_1$*hl*-null mutant led to an increased amount of the CCHL protein (Fig. 3*C*). On the other hand, overexpression of CC_1HL did not suppress the tight respiratory phenotype due to the absence of CCHL, suggesting that CC_1HL cannot promote assembly of holocytochrome *c* in the absence of its cognate lyase CCHL (Fig. 3*A*).

Human and Mouse HCCS Function in the Assembly of Cytochrome c and Cytochrome c$_1$—Only one heme lyase (HCCS) is present in the human genome and its strict specificity toward cytochrome *c* was ascertained from its ability to complement a *cchl*-null mutant and failure to compensate for the lack of CC_1HL (72). This led to the proposal that insertion of heme into apocytochrome c_1 in human is catalyzed by an enzyme other than HCCS or one that is considerably divergent from CC_1HL (72). However, the demonstration in this work that yeast CCHL harbors both CCHL and CC_1HL activities and the fact that only one heme lyase is present in the genomes of multicellular eukaryotes (Fig. 1 in Supplemental Materials) suggest a broader cytochrome specificity for HCCS. We therefore decided to re-examine the role of the single HCCS in *c*-type cytochrome maturation. We expressed the human cDNA encoding HCCS in heme lyase-deficient mutants and tested its capacity to compensate for respiratory deficiency and restore *c*-type holocytochrome assembly. As shown in Fig. 3*A*, the respiratory growth deficiency of mutants lacking either or both heme lyases was complemented upon expression of the human HCCS. An estimate of the functional complementation by determination of the doubling time indicated that human HCCS substitutes equally well for the absence of either or both heme lyases (see Table II). Notably, heterologous or homologous

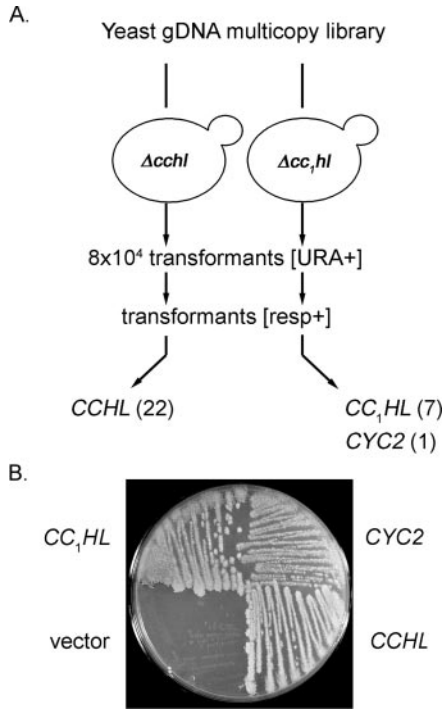

FIG. 4. **Isolation of *CYC2*, a multicopy suppressor of the Δ*cc*$_1$*hl* mutant.** *Panel A*, schematic representation of the multicopy suppressor strategy. The Δ*cchl* (SMY4) and Δ*cc*$_1$*hl* (SMY1) strains were transformed by a wild-type yeast genomic DNA library in multicopy plasmid pFL44L. [*URA*+] transformants were selected on minimal glucose medium lacking uracil, replica-plated on glycerol media, and incubated for 4 days at 28 °C. 22 and 8 [*resp*+] transformants that are restored for the respiratory growth were obtained from Δ*cchl* and Δ*cc*$_1$*hl* strains, respectively. *Panel B*, compensation for the respiratory deficiency by *CYC2* multicopy suppressor. The Δ*cc*$_1$*hl* (YCT1–11B) transformants carrying pFL44L (*vector*), pFL44L-*CC*$_1$*HL*, pFL44L-*CCHL*, or pFL44L-*CYC2* were grown on glucose, replicated on glycerol medium, and incubated 5 days at 28 °C.

complementation of each heme lyase mutant resulted in similar recovery of respiratory proficiency (see Table II). Nevertheless, although HCCS can fully compensate for the defect in cytochrome c_1 assembly, it can only partially alleviate holocytochrome *c* deficiency (Fig. 3*B*). Therefore, in contrast to cytochrome c_1 (see above), it seems that the level of holocytochrome *c* is not limiting for the respiratory activity. We found that, like the human HCCS, the single mouse HCCS also functions in the assembly of both cytochrome *c* and cytochrome c_1 (data not shown). We think that the contrast between our results and those of Schwarz and Cox (72) is not because of a difference in the mutant strain used for the complementation experiments as we verified that the human HCCS can also restore the respiratory growth to the *cc*$_1$*hl*-disrupted allele used in their study (data not shown). Instead, the difference in the expression system chosen for the functional complementation experiments (*S. cerevisiae PGK* promoter *versus Schizosaccharomyces pombe ADH* promoter) could explain the discrepancy we observed. We conclude from our study that human and mouse HCCS display a broader cytochrome specificity than do the yeast orthologs.

Isolation of CYC2, a Multicopy Suppressor of the Absence of CC$_1$*HL*—The finding that broader substrate specificity exists for human and mouse heme lyases or can be enhanced by overexpression of CCHL raises the question of the existence of additional yeast mitochondrial factors that can modulate CCHL and CC_1HL cytochrome specificity. To reveal potential modifiers of the heme lyase activity, we undertook a search for multicopy suppressors able to alleviate the respiratory deficiency of Δ*cchl* or Δ*cc*$_1$*hl* (Fig. 4*A* and "Experimental Procedures"). 22 and 8 respiratory-competent transformants were selected after transformation of the Δ*cchl* and Δ*cc*$_1$*hl* strains, respectively. Diagnostic PCR amplification using *CCHL* and *CC*$_1$*HL* specific primers showed that all of the 22 transformants carried a plasmid containing the CCHL-encoding gene, whereas of the group of 8 transformants, 7 harbored a plasmid with the CC_1HL-encoding gene (data not shown). The remaining Δ*cc*$_1$*hl* transformant displayed a slow growth phenotype and PCR amplification failed to reveal the presence of *CC*$_1$*HL* or *CCHL* genes in the transforming plasmid. We analyzed this plasmid by sequencing the ends of the genomic insert. A search in the yeast genome data base indicated that the plasmid carried a 3.5-kb colinear fragment of chromosome XV that encompassed the *CYC2* ORF encoding a mitochondrial protein with a proposed role in apocytochrome *c* import (43). A plasmid containing solely the entire *CYC2* gene retained the Δ*cc*$_1$*hl* suppression activity but was unable to suppress the Δ*cchl* and Δ*cchl* Δ*cc*$_1$*hl* mutants (Fig. 4*B* and data not shown).

We were unable to monitor the level of *c*-type cytochromes by spectral analysis owing to the fact that overexpression of Cyc2p appears to be toxic to the cells (data not shown). Nevertheless, it is reasonable to assume that the level of holocytochrome c_1 in the *cc*$_1$*hl*-null mutant suppressed by overexpression of Cyc2p is above the 5% wild type level of holoprotein detected in the *cc*$_1$*hl*-null mutant (Fig. 2) because we have established through this study that restoration of the respiratory growth of the *cc*$_1$*hl*-null mutant results systematically in enhanced levels of holocytochrome c_1 (see below and Fig. 5).

Interestingly, a BLAST search identified a putative FAD-binding motif in the COOH-terminal region of Cyc2p ($E = 2 \times 10^{-16}$), predicted to be exposed in the intermembrane space. As already reported (43), we have corroborated that inactivation of the *CYC2* gene does not abolish the respiratory growth and results only in a marginal decrease of spectral holocytochrome *c* (data not shown). The finding that a *cc*$_1$*hl*-null *cyc2*-null mutant exhibits a synthetic tight respiratory block that is no longer suppressed by overexpression of CCHL solidifies the placement of Cyc2p in the CCHL-dependent assembly pathway of cytochrome c_1 (data not shown). That multicopy suppression of Cyc2p does not result in overexpression of CCHL speaks in favor of a distinct mechanism of suppression for Cyc2p (Fig. 3*C*). This also strengthens the view that Cyc2p suppression operates by modulating the CCHL activity.

Recessive and Dominant Genetic Suppressors Compensate for the Absence of CC$_1$*HL*—In search of other genetic interactions in the CCHL pathway, we used a second approach based on the isolation of suppressor mutations able to by-pass the absence of CCHL or CC_1HL and compensate for the respiratory deficiency. We were unable to isolate any suppressors from the Δ*cchl* mutant but spontaneous and UV-induced suppressors of the absence of CC_1HL were recovered. The degree of restoration of respiratory growth at 28 and 36 °C (data not shown) and the genetic nature of the suppressor mutation were used as criteria in the choice of the two spontaneous (R2, R19) and nine UV-induced suppressors (UV9, -14, -16, -17, -18, -34, -43, -48, -50) that we analyzed further (see Table III). Spontaneous suppressors harbor nuclear recessive mutations, whereas UV-induced suppressors carry nuclear dominant mutations. Genetic analysis concluded that all dominant suppressor mutations lay in two distinct unlinked loci (I and II). Locus I was defined by the suppressor mutations *SU14, -16*, *-17*, *-18*, *-34*, *-43*, *-48*, and *-50*, whereas locus II was defined only by the *SU9* allele. Heme stain of cytochrome c_1 and spectral analysis on a subset of suppressed strains showed that recessive and dominant suppressor mutations resulted in partial restoration of

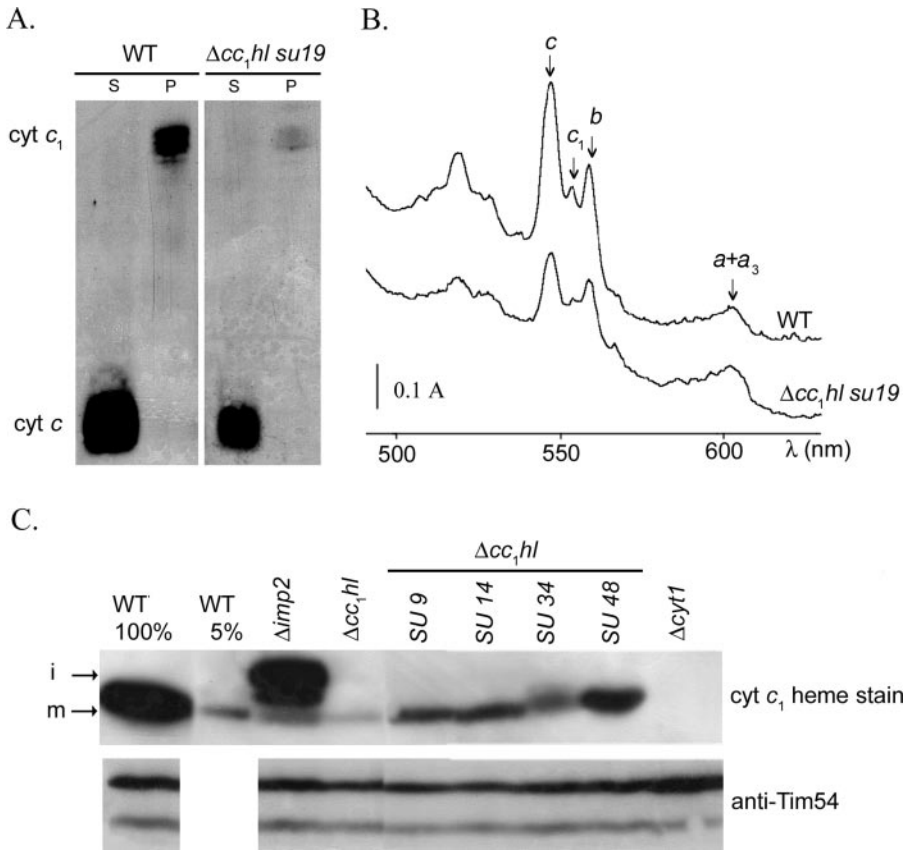

FIG. 5. **Restoration of holocytochrome c_1 in the suppressor strains.** Strains used were: WT (W303-1A), Δ*cc*$_1$*hl* (SMY1), Δ*cyt1* (PHT3), Δ*imp2* (JNY33), Δ*cc*$_1$*hl su19* (R19), Δ*cc*$_1$*hl SU9* (UV9), Δ*cc*$_1$*hl SU14* (UV14), Δ*cc*$_1$*hl SU34* (UV34), and Δ*cc*$_1$*hl SU48* (UV48). *Panel A*, accumulation of mitochondrial *c*-type cytochromes in the Δ*cc*$_1$*hl su19* suppressor. 300 μg of mitochondrial protein purified from cells grown on galactose medium was treated with sodium carbonate. The supernatant (*S*; soluble fraction) and pellet (*P*; membrane fraction) were separated in 12.5% LDS-polyacrylamide gels and transferred to nitrocellulose filters. Heme staining of holocytochrome *c* and c_1 was performed using the Super Signal West Pico (Pierce). Equal loading of the samples was confirmed by red Ponceau staining of the membrane (not shown). *Panel B*, cytochrome spectra of the Δ*cc*$_1$*hl su19* suppressor strain. Cytochrome absorption spectra of galactose-grown cells from WT and Δ*cc*$_1$*hl su19* strains were recorded with a Cary 400 spectrophotometer (81). The absorption maxima of the α bands of cytochromes *c* (546 nm), c_1 (552 nm), *b* (558 nm), and $a + a_3$ (602 nm) are indicated with *arrows*. *Panel C*, accumulation of holocytochrome c_1 in the suppressor strains. 140 μg of mitochondrial protein from cells grown at 28 °C in galactose medium was analyzed for the abundance of holocytochrome c_1. DTT-treated samples were separated in a 12% LDS-polyacrylamide gel at 4 °C. For an estimation of the cytochrome c_1 abundance, a dilution of the wild type sample was loaded on the gel. Following electrophoresis, the gel was transferred to a polyvinylidene difluoride membrane before heme staining by chemiluminescence and immunodetection with antisera against cytochrome c_1 or Tim54 as a loading control. Heme staining was performed using the Super Signal West Dura (Pierce). Bound anti-Tim54 antibodies were detected with ^{125}I-Protein A. Heme staining and autoradiographic exposures were performed under the same conditions for all samples. *i*, intermediate form of holocytochrome c_1; *m*, mature holocytochrome c_1.

TABLE III
Genetic suppressors of Δ*cc*$_1$*hl*

Suppressor	Genetic nature	Respiratory growth[a]		Remarkable trait[b]	Molecular identification[c]
Δ*cc*$_1$*hl su2*	Recessive	+/−	θ^S	↑ CCHL	ND[d]
Δ*cc*$_1$*hl su19*	Recessive	+	θ^R	↓ Cytochrome *c*	ND
Δ*cc*$_1$*hl SU9*	Dominant	++	θ^R	↓ CCHL	CCHL N**F**$_{130}$**L**N → N**LI**N
Δ*cc*$_1$*hl SU14*	Dominant	++	θ^S		Cytochrome c_1 W**S**$_{16}$H → W**Y**H
Δ*cc*$_1$*hl SU34*	Dominant	++	θ^S		Cytochrome c_1 CA**A**$_{42}$CH → CA**P**CH
Δ*cc*$_1$*hl SU48*	Dominant	++	θ^S		Cytochrome c_1 CA**A**$_{42}$CH → CA**D**CH

[a] Growth on glycerol solid medium. In this classification. WT and Δ*cc*$_1$*hl* growth correspond to +++ and ε, respectively. θ^S and θ^R indicate the thermosensitivity (S) or the thermoresistance (R) of strains grown on glycerol solid medium at 37 °C. WT strain is θ^R and Δ*cc*$_1$*hl* is θ^S.
[b] Down accumulation (↓) or up accumulation (↑) of CCHL or cytochrome *c*, as observed in Fig. 7.
[c] The bold letter corresponds to the residue that is mutated in the suppressor. The position of the amino acid replacement is indicated in subscript. Position 1 in cytochrome c_1 corresponds to the first amino acid of the mature protein.
[d] ND, not determined.

holocytochrome c_1 compared with the residual level detected in the Δ*cc*$_1$*hl* mutant (Fig. 5 and data not shown).

Alteration of the Expression of Cytochrome c or CCHL in the Recessive Suppressors—Surprisingly, restoration of holocytochrome c_1 assembly by the suppressor mutation *su19* coincided with loss of holocytochrome *c* accumulation (Fig. 5, *A* and *B*). Because of the genetic nature of the suppressor mutation, it is conceivable that loss of cytochrome *c* is the recessive trait causing restoration of holocytochrome c_1. Two possible mechanisms accounting for the down accumulation of cytochrome *c* through the mutation *su19* can be envisaged. One hypothesis is that the mutation acts at the transcriptional/post-transcriptional level by regulating the levels of cytochrome *c* structural mRNAs. Another possibility is that a translational or post-translational step of the synthesis of cytochrome *c* is affected by the suppressor mutation. To discriminate between these two

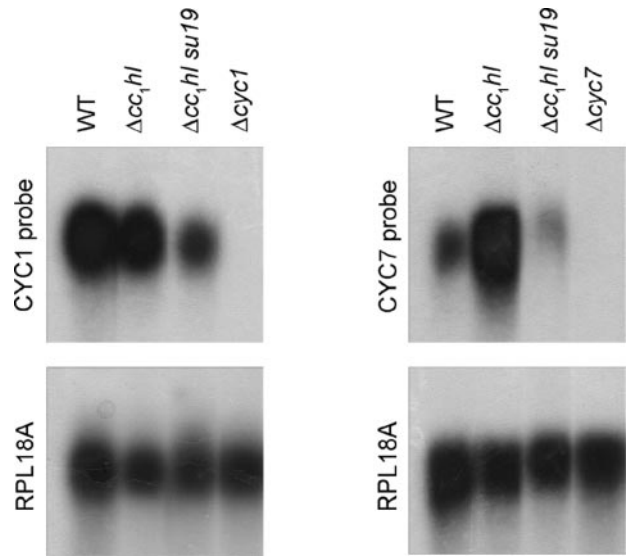

FIG. 6. **Reduced accumulation of *CYC1* and *CYC7* mRNAs in the Δ*cc*$_1$*hl su19* suppressor.** RNA was extracted from wild type (WT) (W303-1A), Δ*cc*$_1$*hl* (SMY1), Δ*cc*$_1$*hl su19* (R19), Δ*cyc1* (YPH9-2A), and Δ*cyc7* (W303ΔCYC7), and analyzed as described under "Experimental Procedures" using [α-^{32}]dCTP-labeled *CYC1*, *CYC7*, or *RPL18A* probes. Autoradiographic exposures using an amplifying screen were 4 days for the *CYC1* probe, 8 days for the *CYC7* probe, and 2 days for the *RPL18A* probe.

hypotheses, we performed RNA blot hybridization and assessed the accumulation of *CYC1* and *CYC7* mRNAs encoding isoforms 1 and 2 of holocytochrome *c*, respectively (Fig. 6). It appears that both *CYC1* and *CYC7* mRNA levels were significantly diminished in the Δ*cc*$_1$*hl su19* suppressor strain. Interestingly, the absence of CC_1HL led to an increase in the accumulation of the *CYC7* transcript, whereas the abundance of the *CYC1* transcript was not affected. Up-regulation of the *CYC7* mRNA probably accounts for the increase in the level of holocytochrome *c* that we have observed in the cytochrome spectra of the *cc*$_1$*hl*-null allele (see Fig. 2*A*). We concluded from this experiment that the decrease of cytochrome *c* in the Δ*cc*$_1$*hl su19* suppressor is caused by a reduction in the abundance of the *CYC1* and *CYC7* mRNAs. Because our interest lies primarily in the study of the post-translational steps of *c*-type cytochrome synthesis, we did not pursue the molecular identification of the *su19* mutation.

The hypothesis that the suppressor mutation *su2* may lie within the CCHL-encoding gene was inferred from the slight increase (≃2-fold) in the accumulation of CCHL in the Δ*cc*$_1$*hl su2* strain (Fig. 7). However, molecular sequencing of the entire *CCHL* ORF and 1 kb upstream of the initiation codon did not reveal any mutation and negated this hypothesis. We did not devote efforts toward the identification of the suppressor mutation *su2* in the course of this study based on the fact that, similarly to multicopy suppression by CCHL, we believed that *su2* mediates suppression through an enhanced level of CCHL.

Dominant Suppressor Mutations Map to the Cytochrome c_1 and CCHL-encoding Genes—To identify the dominant suppressor mutations at the molecular level, we carried out a candidate gene approach based on our prediction of the mechanism of suppression. *A priori*, we assumed that restoration of holocytochrome c_1 assembly resulted from enhanced specificity of the CCHL for its non-cognate substrate cytochrome c_1. This could occur through altered specificity of the enzyme (CCHL) for its non-cognate substrate (cytochrome c_1) or modulation of CCHL activity by Cyc2p or unknown modifiers.

Based on the observation that both CCHL and cytochrome *c* abundance are affected by the suppressor mutation *SU9* (Fig. 7), we suspected that this mutation mapped to the *CCHL* gene. We tested this hypothesis through genetics by performing a recombination test, crossing the Δ*cc*$_1$*hl SU9* suppressor with a Δ*cchl* mutant. The observation that all the single mutant Δ*cc*$_1$*hl* spores that we examined displayed a suppressed phenotype (*i.e.* restored respiratory growth in the absence of CC_1HL) led us to conclude that locus I is linked to the CCHL-encoding gene. We sequenced the entire *CCHL* gene in the Δ*cc*$_1$*hl SU9* suppressor and confirmed the presence of two contiguous mutations (TTTCTA to CT**AA**TA) changing the FL residues in the coding sequence to LI (see Fig. 1 of Supplemental Materials).

Similarly, we deduced that locus II is linked to the *CYT1* gene encoding apocytochrome c_1 on the basis that all the single mutant Δ*cc*$_1$*hl* spores from a cross between a Δ*cc*$_1$*hl SU34* strain and a Δ*cyt1* mutant exhibited a suppressed phenotype. Sequencing of the *CYT1* ORF in all the allelic suppressor strains confirmed the genetic results at the molecular level. Three mutations (referred to as *CYT1–14*, *-34*, and *-48*) were found. Two of them (*CYT1–34* and *-48*) lay in the heme binding motif of cytochrome c_1 (CAACH) and one (*CYT1–14*) occurred in a conserved region of the protein, 24 residues upstream of the heme-binding site (see Fig. 2 of Supplemental Materials). The *SU14* allele resulted in a serine (TCC) to tyrosine (T**A**C) change, *SU16*, *-18*, *-34*, *-43*, and *-50* corresponded to an alanine (GCC) to proline (**C**CC) substitution and *SU17* and *-48* led to an alanine (GCC) to aspartic (G**A**C) mutation. We concluded from the study of the dominant suppressors that mutations in the substrate (cytochrome c_1) or the enzyme (CCHL) were able to change the specificity of the cytochrome/heme lyase interaction.

DISCUSSION

Whereas considerable detail is known of bacterial and plastid cytochrome *c* maturation, the biogenesis of mitochondrial *c*-type cytochromes (system III) has remained so far poorly understood despite the fact it was the first cytochrome assembly pathway to be described (8, 29, 40). In this paper, we have re-examined the participation of its central components, namely the CCHL and CC_1HL, using a genetic approach in yeast as an experimental model system. We show that (i) yeast CCHL and the single heme lyase in human and mouse are active toward both cytochrome *c* and c_1, whereas the specificity of yeast CC_1HL is indeed restricted only to cytochrome c_1, (ii) yeast CCHL activity toward apocytochrome c_1 is enhanced by increasing the expression of CCHL, decreasing the expression of cytochrome *c*, or through missense mutations in CCHL or cytochrome c_1, and (iii) Cyc2p, a putative flavoprotein, is required for the activity of CCHL.

Overlapping Specificities in the Heme Lyase Family—Earlier work in the yeast system led to the proposal that CCHL and CC_1HL are distinct in their substrate specificity in that each heme lyase can only convert its own apocytochrome substrate into the holoform (30–32, 39). However, our work provides several lines of evidence that *in vivo*, both cytochrome *c* and c_1 can be acted upon by a single heme lyase. First, we have observed that the deletion of the entire *CC*$_1$*HL* gene does not lead to a complete block of the respiratory activity indicating that a functionally overlapping factor must display some CC_1HL activity. Then, we have demonstrated that the *cc*$_1$*hl*-null mutant synthesizes low levels of holocytochrome c_1 that are attributable to the weak activity of CCHL and account for the residual respiratory growth of the mutant. This result proves unambiguously that CCHL is intrinsically able to act upon both apocytochrome *c* and apocytochrome c_1. That the broader cytochrome specificity of heme lyase is not a peculiarity of yeast but extends to other organisms is deduced from our complementation experiments where we demonstrate that the

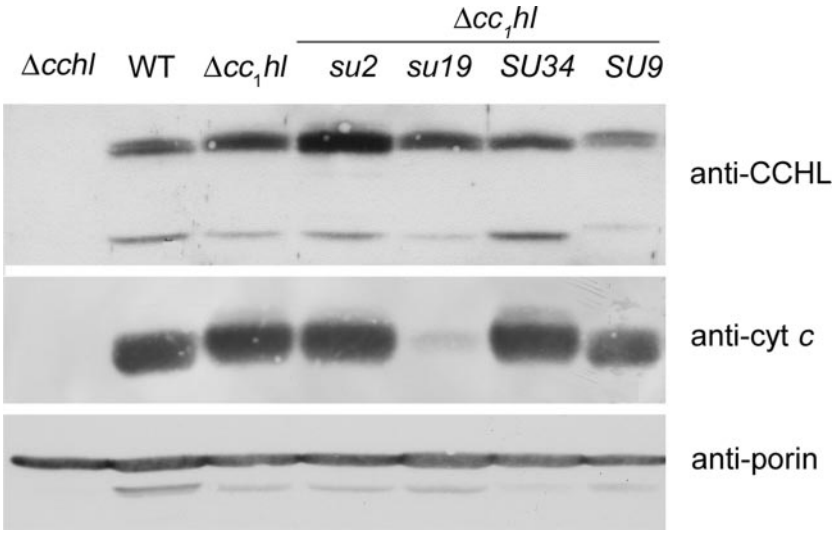

FIG. 7. **Accumulation of CCHL and cytochrome *c* in suppressor strains.** Strains used were: wild type (*WT*) (W303-1A), Δcc_1hl (SMY1), Δ*cchl* (SMY4), Δcc_1hl *su2* (R2), Δcc_1hl *su19* (R19), Δcc_1hl *SU34* (UV34), and Δcc_1hl *SU9* (UV9). 160 μg of mitochondrial protein from cells grown at 28 °C in galactose medium was separated on a 16% acrylamide, 0.5% bisacrylamide SDS gel and transferred to nitrocellulose membrane. Immunodetection was carried out with antisera against CCHL, cytochrome *c* and porin (loading control), and horseradish-conjugated secondary antibody.

single human and mouse HCCS carries both CCHL and CC_1HL activities. This indicates that in multicellular eukaryotes cytochrome *c* and cytochrome c_1 are presumably assembled via the activity of one heme lyase.

Strict Specificity of the Yeast CC_1HL—The complete lack of cytochrome c in the yeast *cchl*-null mutant indicates that cytochrome *c* cannot be acted upon *in vivo* by CC_1HL. One possibility is that CC_1HL cannot promote holocytochrome *c* assembly in the absence of CCHL because of the lack of import of apocytochrome *c* in the mitochondria. Indeed, in addition to facilitating covalent heme attachment, CCHL unlike CC_1HL is also known to play a role in the import of its own apocytochrome substrate (73, 74). However, the delivery of cytochrome *c* to the mitochondria through the cytochrome c_1 import pathway by fusing at its amino terminus the bipartite targeting sequence of apocytochrome c_1 does not result in conversion of the fusion protein to a holoform (75). It is thus probable that cytochrome *c* cannot be a substrate of CC_1HL because the proper recognition elements for apocytochrome *c* are normally lacking in CC_1HL. Furthermore, the observation that no multicopy or genetic suppressors able to substitute for the absence of CCHL could be isolated reinforces the view that the specificity of CC_1HL toward apocytochrome c_1 is strict and cannot be altered in favor of its non-cognate substrate apocytochrome *c*. Nevertheless, it is possible that mutations can convert CC_1HL into a CCHL but could not be revealed in our screen because of their deleterious effect on holocytochrome c_1 assembly. Alternatively, more than one mutational event might be required for CC_1HL to act upon apocytochrome *c*.

Alterations of the Expression of CCHL or Cytochrome c Favor CCHL Activity Toward Cytochrome c_1—Increased accumulation of CCHL through multicopy or genetic suppression yields enhanced activity of CCHL toward its non-cognate substrate apocytochrome c_1. Interestingly, we found that a modest increase (~2-fold) in the abundance of CCHL can suppress the absence of CC_1HL in the *su2* suppressor. This is consistent with the observation that suppression of the cc_1hl-null mutant also occurs upon expression of CCHL from a centromeric vector (data not shown) and suggests that only a slight modification of CCHL abundance is sufficient to compensate for the absence of CC_1HL. This slight increase may generate some excess CCHL that is not already bound to apocytochrome *c* and therefore can act on apocytochrome c_1. This view is corroborated by the fact that reduced accumulation of the cytochrome *c* substrate in a cc_1hl-null background leads to an increase in holocytochrome c_1 accumulation. This suggests that, in the absence of CC_1HL, holocytochrome c_1 assembly is favored whenever there is a decrease in the ratio of apocytochrome *c* to CCHL. In accord with this model, it is conceivable that up-regulation of the *CYC7* mRNA in the cc_1hl strain results in an increase in apocytochrome *c* that favors the activity of CCHL toward its preferred substrate apocytochrome *c* over the activity toward non-cognate apocytochrome c_1.

Mutations in CCHL or Cytochrome c_1 Increase CCHL Activity Toward Cytochrome c_1—The view that cytochrome c_1 is a substrate for the heme lyase reaction catalyzed by CCHL is further substantiated by the isolation of dominant mutations in the *CYT1* and *CCHL* genes that are able to by-pass the lack of CC_1HL. Because wild type CCHL has the intrinsic ability to function as a CC_1HL, it is conceivable that the suppressor mutations modify residues involved in the interaction between CCHL and apocytochrome c_1 and improve affinity of the enzyme for the apocytochrome c_1 substrate. Limited information is available regarding the sequence requirements for recognition of apocytochrome *c* and c_1 by their heme lyases. It is only known that partially purified yeast CCHL can attach heme to fruit fly and horse apocytochrome *c* and also to a synthetic peptide corresponding to the first 25 residues of horse cytochrome *c* in an *in vitro* reaction (42, 76). Because the amino acid sequences of fruit fly, horse, and yeast cytochrome *c* differ considerably, it is possible that CCHL may recognize only a limited region of the apoprotein presumably contained in the sequence encompassed by the 25-residue peptide. However, the failure of alleles of iso-2 cytochrome *c* containing small deletions in the COOH-terminal part to undergo covalent heme attachment *in vivo*, despite normal import in the mitochondria, seems to indicate that the requirement for CCHL action is much more stringent in the *in vivo* mitochondrial context (77).

That seven suppressor alleles corresponding to two classes of mutation map to the second alanine of the CAACH motif underscores the importance of the heme-binding site of apocytochrome c_1 as a recognition site for CCHL *in vivo*. Mutation of the serine residue located 24 residues upstream of the C*XX*CH motif into a tyrosine in the *CYT1–14* allele points to another potential interaction site for CCHL (see Fig. 2 of Supplemental Materials). On the other hand, only one suppressor allele (*CYC3–9*) in the *CCHL* gene was identified through the mutagenesis screen and similarly to the mutations in cytochrome c_1, we hypothesize that this mutation modifies the interaction of CCHL to favor cytochrome c_1. There is the possibility that other sites of interaction in CCHL and cytochrome c_1 exist but could not be uncovered because our screen is based on restoration of respiratory activity that relies on both functional holocytochrome *c* and c_1.

Cyc2p, a Putative Flavoprotein in the Heme Lyase Pathway—Previous investigations have led to the conclusion that the *CYC2* gene product is a membrane component required for normal mitochondrial integrity. Loss of Cyc2p causes partial defects in various mitochondrial processes including the biogenesis of holocytochrome *c* (31, 43–45). Yeast strains with a complete deletion of the *CYC2* gene display normal levels of holocytochrome c_1 but contain at least 10% of the normal content of holocytochrome *c* and accumulate apocytochrome *c* in the cytoplasm. To explain the effects of *cyc2* mutations on holocytochrome *c* maturation, a function of Cyc2p in the translocation of apocytochrome *c* or as an accessory factor in the heme attachment reaction was initially postulated (31, 40, 43). Our discovery that overexpression of Cyc2p can restore holocytochrome c_1 assembly in the absence of CC_1HL reveals that the function of this protein is not limited to the biogenesis of holocytochrome *c* but also extends to that of holocytochrome c_1. We hypothesized that Cyc2p suppresses the absence of CC_1HL by modulating the activity of CCHL in favor of its non-cognate substrate apocytochrome c_1. The fact that we did not retrieve CCHL as a multicopy suppressor of Δcc_1hl indicates that our screen for suppressors is not saturated and that other modulators of CCHL activity could still be revealed. That Cyc2p interacts functionally with CCHL and participates in the heme attachment reaction is inferred from our finding that a *cyc2*-null cc_1hl-null mutant displays a synthetic respiratory block that can no longer be suppressed by overexpression of CCHL or the suppressor mutations in CCHL or cytochrome c_1. Therefore, whereas the function performed by Cyc2p in cytochrome *c* biogenesis appears redundant in a wild type background, it becomes essential when CC_1HL is inactive. The mechanism by which Cyc2p could control the activity of CCHL remains unclear but the identification of a putative FAD binding motif, distinctive for the FAD fold of the ferredoxin reductase family (78) and predicted to be exposed in the intermembrane space, suggests a function in the redox chemistry of the heme lyase reaction. The need for redox chemistry to maintain the heme and apocytochrome substrates in a reduced state in the heme attachment reaction has been under investigated in system III because of the belief that the mitochondrial intermembrane space was a reducing compartment. Yet, the previous biochemical studies by Neupert and co-workers (38, 79) had established the necessity for redox cofactors NADH and flavin nucleotides in the heme attachment reaction to apocytochrome *c* and apocytochrome c_1 *in organello*. This was also confirmed recently *in vitro* by the discovery that the reaction of mitochondrial apocytochrome *c* with heme yields thioether bond formation only when the appropriate redox conditions are provided (80). Moreover, cytochrome *c* assembly pathways in bacteria and plastids have been shown genetically to have a definite requirement for redox chemistry (9, 21, 23). We therefore believe that the *in vivo* oxidation state of the cysteine residues in mitochondrial apocytochrome *c* and c_1 is also under the control of as yet unidentified components. An attractive hypothesis is that Cyc2p operates in a redox subpathway in the context of the heme lyase reaction. It remains to be demonstrated that Cyc2p is a flavoprotein and to establish the link between co-factor binding and the activity of the protein in the CCHL pathway. We are currently investigating the function of Cyc2p to define the participation of this unique assembly factor in the heme lyase pathway.

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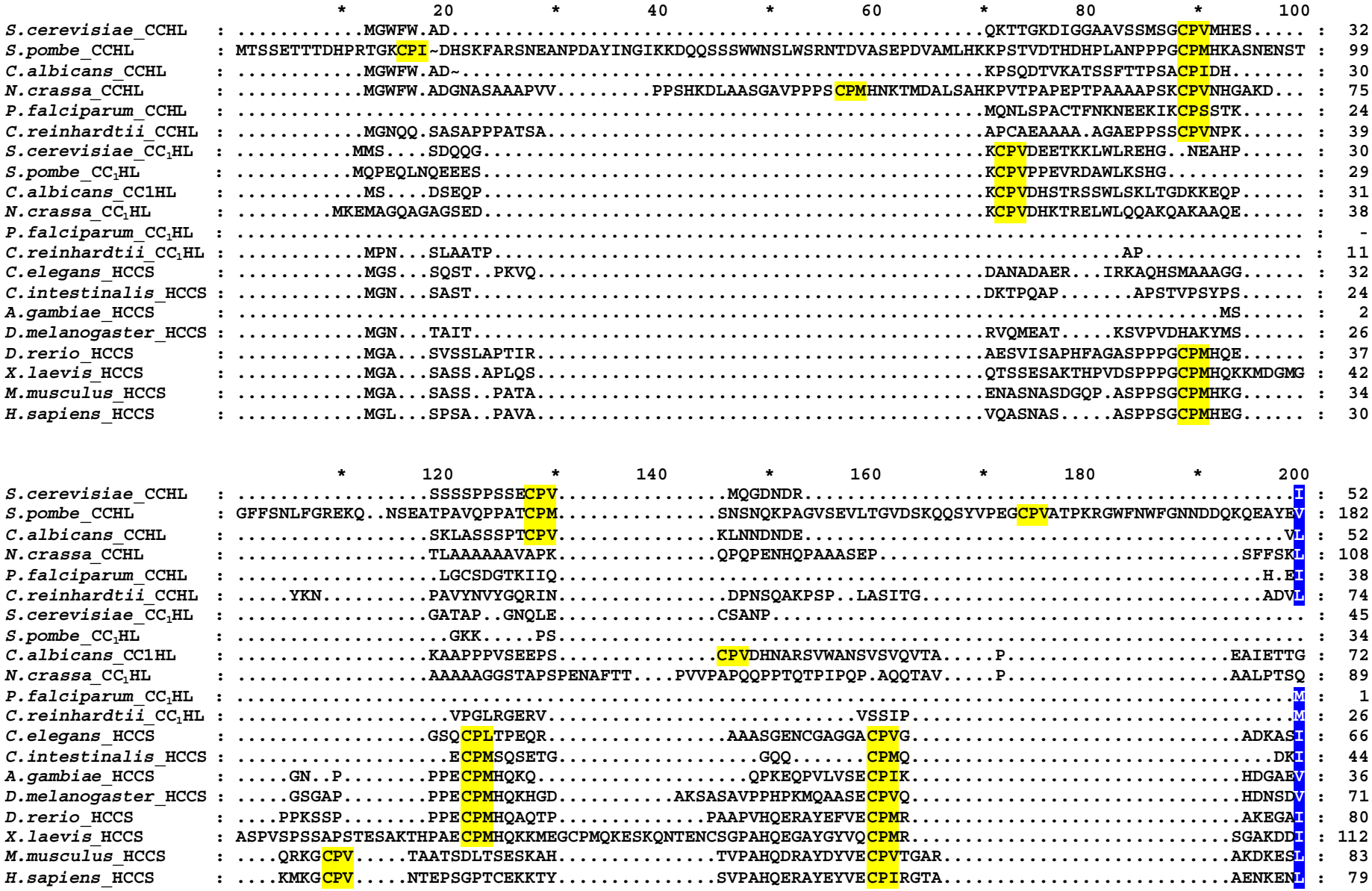
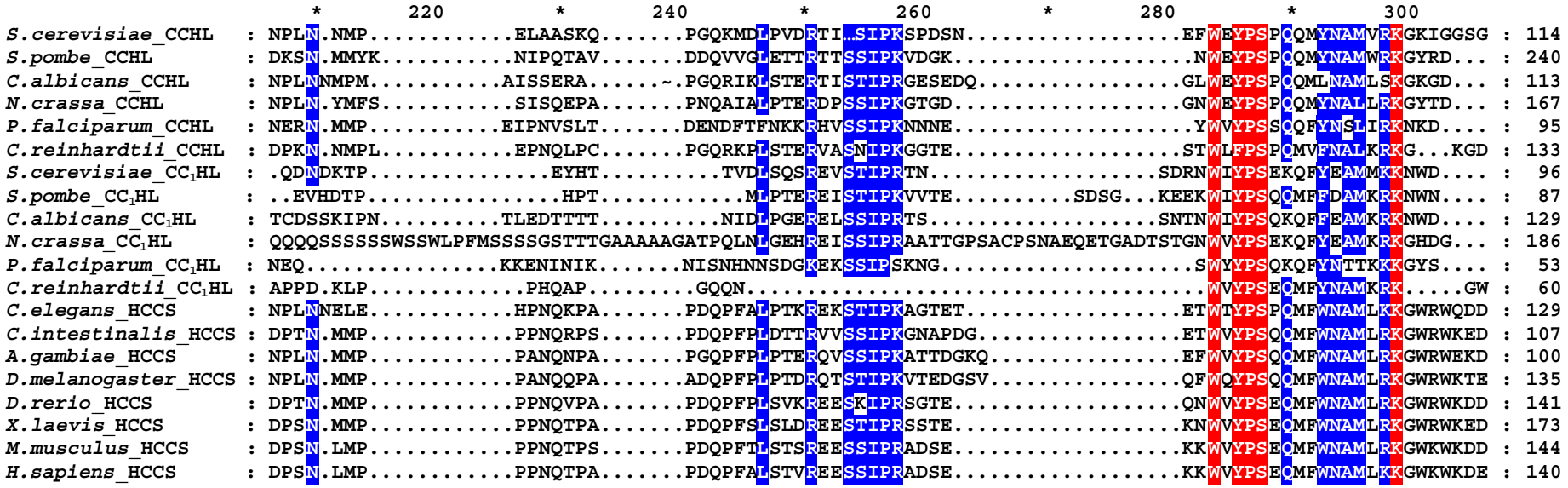
Bernard *et al.*, Figure 1 Supplemental data



Bernard *et al.*, Figure 1 Supplemental data



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                          *       420         *       440         *       460         *       480         *       500
S.cerevisiae_CCHL       : ........SHFSQ.ELPFDRHDWIVLRGERKAEQQPPTFKEVRYVLDFYGGPD.DENGM..PTFHVDVRPALDSLDNAKDRMTRFLDRMISGPSSSSSAP : 269
S.pombe_CCHL            : ........NRWGSGEGPFDRHDWYVQR....KDNS.....IVRYVIDYYEAPD.SADGK..PVFSLDVRPAVDSFESVALRWKHWRAMRQMQQQ..... : 377
C.albicans_CCHL         : ........ETFNT.IPPFDRHDWTVLRSCGRNQGW....KEVRYVIDYYGAPDDEEAGM..PAFMLDTRPALDNLTNARDRFTHWAYPLWKKAMG.EVRD : 264
N.crassa_CCHL           : ........SKYAT.EPPFDRHDWYVSR..DENGQK....KEVRYVIDFYSAPP.EPTGE..PVFYLDVRPAVT.VTGACERLLRWGGDVWWKASGGEVRE : 341
P.falciparum_CCHL       : NNNNKKSVKGFFFMGRPFDRHDWYVNRCG..........TQVKYILDYYN..DESINDD..KNIYIDVRPAMNSFSNVWDRLRYPFYEFYFKYVKKDELF : 249
C.reinhardtii_CCHL      : .........HMLGGPAPFDRHDWVIDRCG..........KEVRYIIDFYFFDDKAGTPQ..AFEIV.ARPAVDSVEAALDRVKM.............. : 253
S.cerevisiae_CC1HL      : .........ILHLAKPFDRHDWQIDRCG..........KTVDYVIDFYSTDLNDANSQQQPLIYLDVRPKLNSFEGFRLRFWKSLGF.......... : 224
S.pombe_CC1HL           : .........LLGYNKPFDRHDWLVNRCG..........RKVAYVIDFYNG..PTVNGT..PSIYLDVRPKL.SVHGAWMRVYRWTNEHFSQNSK..... : 216
C.albicans_CC1HL        : .........IFGYDKPFDRHDWLINRCG..........VEVEYVIDFY.T...GQNSQ....VYLDVRPKLNTLEGIKMRFGRPFGF.......... : 251
N.crassa_CC1HL          : .........LLGYTAPFDRHDWIVDRCG..........TRVDYVIDFYAGRNNDRAGAGKLNFYLDVRPKLNTWEGVKMRALRFVGMN.......... : 317
P.falciparum_CC1HL      : ............YNKPFDRHEWYIDRCG..........NTIKYIIDYYDG.KKEKNSA..VSIYIDARPQLN.HQNAIDNVKIIYIKICRFLNN...LF : 184
C.reinhardtii_CC1HL     : .........NFVGFGLPFDRHDWVVDRCG..........KEVR.................................................... : 142
C.elegans_HCCS          : .........LFLGYDLPFDRHDWIVDRCGT.........KQVQYVIDYYDGGAVDPSSK..LFTILDVRPAVNDIGNIWDRMVVAYWRFKFETLGFTPSL : 268
C.intestinalis_HCCS     : .........SWMGYELPFDRHDWIVDRCG..........KQVRYIIDYYDGGVVDPDTY..QFTLLDVRPALDSFGAFYDRSKATLMRWTSSYKAGGDSR : 245
A.gambiae_HCCS          : .........NMMGYELPFDRHDWIIDRCG..........KDVRYVIDYYDGGMVD.EKY..KFALLDVRPAMDSFDNVWDRMKVAYMRWKFELEDKL... : 233
D.melanogaster_HCCS     : .........SWLGYELPFDRHDWIVDRCG..........KDVRYVIDYYDGGLVD.KDY..RFALLDVRPAMDSVDNVWDRMRVAYMRWKYELFEKFGSA : 271
D.rerio_HCCS            : .........HWMGYELPFDRHDWIVDRCG..........KEVRYVIDYYDGDIDK.DSY..QFSILDVRPAFDSMQAVWDRMKVAWWRWTS......... : 269
X.laevis_HCCS           : .........SWMGYELPFDRHDWIVDRCG..........RKVRYVIDYYDGGEVD.NNY..QFSILDVRPAFDSMGAIWDRMKVAWWRWTS......... : 301
M.musculus_HCCS         : .........SWMGYELPFDRHDWIINRCG..........TEVRYVIDYYDGGEVN.KEY..QFTILDVRPAFDSFSAVWDRMKVAWWRWTS......... : 272
H.sapiens_HCCS          : .........SWMGYELPFDRHDWIINRCG..........TEVRYVIDYYDGGEVN.KDY..QFTILDVRPALDSLSAVWDRMKVAWWRWTS......... : 268

                                *       520         *
S.cerevisiae_CCHL       : ..................................... :   -
S.pombe_CCHL            : ..................................... :   -
C.albicans_CCHL         : ..................................... :   -
N.crassa_CCHL           : RERSK................................ : 346
P.falciparum_CCHL       : K.................................... : 250
C.reinhardtii_CCHL      : ..................................... :   -
S.cerevisiae_CC1HL      : ..................................... :   -
S.pombe_CC1HL           : ..................................... :   -
C.albicans_CC1HL        : ..................................... :   -
N.crassa_CC1HL          : ..................................... :   -
P.falciparum_CC1HL      : ..................................... :   -
C.reinhardtii_CC1HL     : ..................................... :   -
C.elegans_HCCS          : PIPPTEGHNVNH......................... : 280
C.intestinalis_HCCS     : VYHEGSDATNKPNMKTVTHNDSDIPPECPMHAAKS : 280
A.gambiae_HCCS          : ..................................... :   -
D.melanogaster_HCCS     : DGGKVTAGSD........................... : 281
D.rerio_HCCS            : ..................................... :   -
X.laevis_HCCS           : ..................................... :   -
M.musculus_HCCS         : ..................................... :   -
H.sapiens_HCCS          : ..................................... :   -
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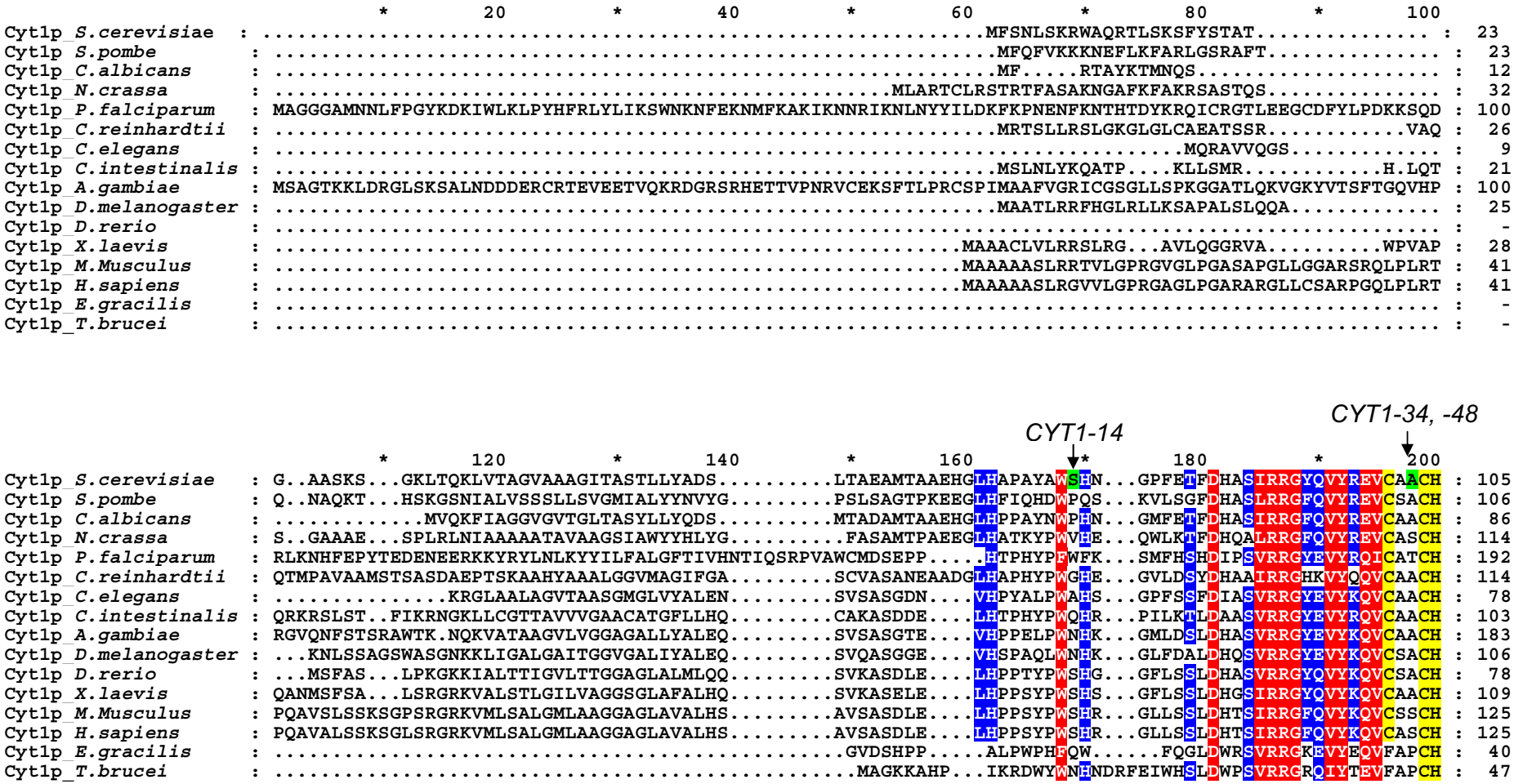
Figure 1 Supplemental data: Conservation of mitochondrial cytochrome *c* and c_1 heme lyases

Sequences of cytochrome *c* heme lyases (CCHL) and cytochrome c_1 heme lyases (CC_1HL) from *Saccharomyces cerevisiae* (CCHL:P06182, CC_1HL:X67017), *Schizosaccharomyces pombe* (CCHL:NP_596655, CC_1HL:NP_594026), *Candida albicans* (CCHL:P53700, [1]CC1HL: CA5141), *Neurospora crassa* (CCHL:P14187, [2]CC_1HL:NCU08138.1), *Plasmodium falciparum* (CCHL:AAN36323, CC_1HL:NP_701401), [3]*Chlamydomonas reinhardtii* (CCHL:genewise.1400.13.1, CC_1HL: genewise.1076.18.1) and holocytochrome *c* synthetase (HCCS) from *Caenorhabditis elegans* (NP_496403), [4]*Ciona intestinalis* (ci0100146451), *Anopheles gambiae* (EAA06088), *Drosophila melanogaster* (NP_651003), *Danio rerio* (AAH44486), *Xenopus laevis* (AAH45005), *Mus musculus* (NP_032248), *Homo sapiens* (NP_005324), were aligned using the CLUSTALW algorithm (Blosum62 scoring matrix) in Bioedit. The alignment was edited using the GeneDoc multiple alignment editor. The tilde character (**~**) indicates manual editing of the alignment. Amino-acids strictly conserved in all sequences are shaded red and those conserved in the majority of the sequences (10 of 20) are shaded blue. The heme-regulatory motifs (HRMs), defined by the sequence CPX (X= V, M, I, L, S) are highlighted in yellow. The position of the residues mutated in the suppressor allele *CYC3-9* is indicated in green.

[1] See *Candida albicans* database at http://genolist.pasteur.fr/CandidaDB/index.html
[2] See *Neurospora crassa* database at http://www-genome.wi.mit.edu/annotation/fungi/neurospora/index.html
[3] See *Chlamydomonas reinhardtii* draft genome at http://genome.jgi-psf.org/chlre1/chlre1.home.html
[4] See *Ciona intestinalis* draft genome sequence at http://fugu.jgi-psf.org/ciona4/ciona4.home.html


CYT1-14
CYT1-34, -48
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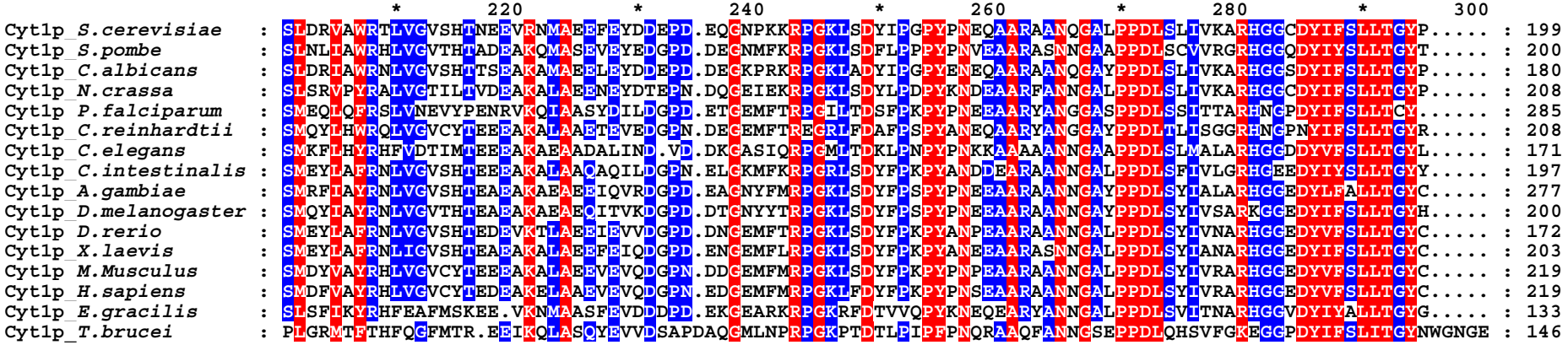
Cyt1p_S.pombe
Cyt1p_C.albicans
Cyt1p_N.crassa
Cyt1p_P.falciparum
Cyt1p_C.reinhardtii
Cyt1p_C.elegans
Cyt1p_C.intestinalis
Cyt1p_A.gambiae
Cyt1p_D.melanogaster
Cyt1p_D.rerio
Cyt1p_X.laevis
Cyt1p_M.Musculus
Cyt1p_H.sapiens
Cyt1p_E.gracilis
Cyt1p_T.brucei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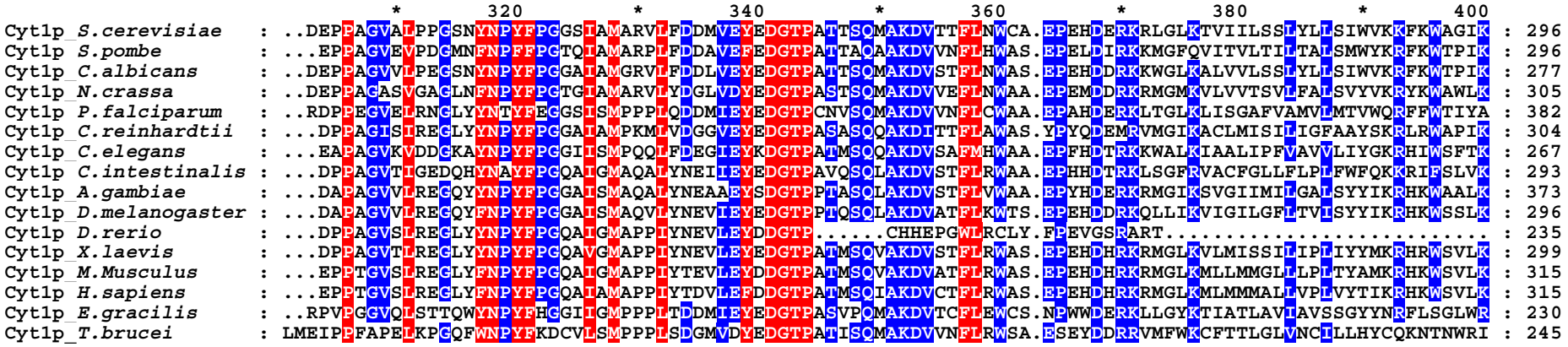
Cyt1p_S.pombe : SLNLIAWRHLVGVTHTADEAKQMASEVEYEDGPD.DECNMFKRPGKLSDFLPPPYPNVEAARASNNGAAPPDLSCVVRGRHGGQDYIYSLLTGYT..... : 200
Cyt1p_C.albicans : SLDRIAWRNLVGVSHTTSEAKAMAEELEYDDEPD.DECKPRKRPGKLADYIPGPYENEQAARAANQGAYPPDLSLIVKARHGGSDYIFSLLTGYP..... : 180
Cyt1p_N.crassa : SLSRVPYRALVGTILTVDEAKALAEENEYDTEPN.DQCEIEKRPGKLSDYLPDPYKNDEAARFANNGALPPDLSLIVKARHGGCDYIFSLLTGYP..... : 208
Cyt1p_P.falciparum : SMEQLQFRSLVNEVYPENRVKQIAASYDILDGPD.ETCEMFTRPGILTDSFPKPYPNEEAARYANGGASPPDLSSITTARFNCPDYIFSLLTCY..... : 285
Cyt1p_C.reinhardtii : SMQYLHWRQLVGVCYTEEEAKALAAETEVEDGPN.DECEMFTREGRLFDAFPSPYANEQAARYANGGAYPPDLTLISGGRFNCPNYIFSLLTGYR..... : 208
Cyt1p_C.elegans : SMKFLHYRHFVDTIMTEEEAKAEAADALIND.VD.DKCASIQRPGMLTDKLPNPYPNKKAAAAANNGAAPPDLSLMALARHGCDDYVFSLLTGYL..... : 171
Cyt1p_C.intestinalis : SMEYLAFRNLVGVSHTEEEAKALAAQAQILDGPN.ELCKMFKRPGRLSDYFPKPYANDDEARAANNGALPPDLSFIVLGRHGEEDYIYSLLTGYY..... : 197
Cyt1p_A.gambiae : SMRFIAYRNLVGVSHTEAEAKAEAEEIQVRDGPD.EACNYFMRPGKLSDYFPSPYPNEEAARAANNGAYPPDLSYIALARHGGEDYLFALLTGYC..... : 277
Cyt1p_D.melanogaster : SMQYIAYRNLVGVTHTEAEAKAEAEQITVKDGPD.DTCNYYTRPGKLSDYFPSPYPNEEAARAANNGAYPPDLSYIVSARKGGEDYIFSLLTGYH..... : 200
Cyt1p_D.rerio : SMEYLAFRNLVGVSHTEDEVKTLAEEIEVVDGPD.DNCEMFTRPGKLSDYFPKPYANPEAARAANNGALPPDLSYIVNARHGGEDYVFSLLTGYC..... : 172
Cyt1p_X.laevis : SMEYLAFRNLIGVSHTEAEAKALAEEFEIQDGPD.ENCEMFLRPGKLSDYFPKPYANEEAARASNNGALPPDLSYIANARHGGEDYIFSLLTGYC..... : 203
Cyt1p_M.Musculus : SMDYVAYRHLVGVCYTEEEAKALAEEVEVQDGPN.DDCEMFMRPGKLSDYFPKPYPNPEAARAANNGALPPDLSYIVRARHGGEDYVFSLLTGYC..... : 219
Cyt1p_H.sapiens : SMDFVAYRHLVGVCYTEDEAKELAAEVEVQDGPN.EDCEMFMRPGKLFDYFPKPYPNSEAARAANNGALPPDLSYIVRARHGGEDYVFSLLTGYC..... : 219
Cyt1p_E.gracilis : SLSFIKYRHFEAFMSKEE.VKNMAASFEVDDDPD.EKCEARKRPGKRFDTVVQPYKNEQEARYANNGALPPDLSVITNARHGGVDYIYALLTGYG..... : 133
Cyt1p_T.brucei : PLGRMTFTHFQGFMTR.EEIKQLASQYEVVDSAPDAQCMLNPRPGKPTDTLPIPFPNQRAAQFANNGSEPPDLQHSVFGKEGGPDYIFSLITGYNWGNGE : 146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Cyt1p_S.pombe : ...EPPAGVEVPDGMNFNPFFPGTQIAMARPLFDDAVEFEDGTPATAQAAKDVVNFLHWAS.EPELDIRKKMGFQVITVLTILTALSMWYKRFKWTPIK : 296
Cyt1p_C.albicans : ..DEPPAGVVLPEGSNYNPYFPGGAIAMGRVLFDDLVEYEDGTPATTSQMAKDVSTFLNWAS.EPEHDDRKKWGLKALVVLSSLYLLSIWVKRFKWTPIK : 277
Cyt1p_N.crassa : ..DEPPAGASVGAGLNFNPYFPGTGIAMARVLYDGLVDYEDGTPASTSQMAKDVVEFLNWAA.EPEMDDRKRMGMKVLVVTSVLFALSVYVKRYKWAWLK : 305
Cyt1p_P.falciparum : ..RDPPEGVELRNGLYYNTYFEGGSISMPPPLQDDMIEYEDGTPCNVSQMAKDVVNFLCWAA.EPAHDERKLTGLKLISGAFVAMVLMTVWQRFFWTIYA : 382
Cyt1p_C.reinhardtii : ...DPPAGISIREGLYYNPYFPGGAIAMPKMIVDGGVEYEDGTPASASQQAKDITTFLAWAS.YPYQDEMRVMGIKACLMISILIGFAAYSKRLRWAPIK : 304
Cyt1p_C.elegans : ...EAPAGVKVDDGKAYNPYFPGGIISMPQQIFDEGIEYKDGTPATMSQQAKDVSAFMHWAA.EPFHDTRKKWALKIAALIPFVAVVLIYGKRHIWSFTK : 267
Cyt1p_C.intestinalis : ...DPPAGVTIGEDQHYNAYFPGQAIGMAQALYNEIIEYEDGTPAVQSQLAKDVSTFLRWAA.EPHHDTRKLSGFRVACFGLLFLPLFWFQKKRIFSLVK : 293
Cyt1p_A.gambiae : ...DAPAGVVLREGQYYNPYFPGGAISMAQALYNEAAEYSDGTPPTASQLAKDVSTFLVWAA.EPYHDERKRMGIKSVGIIMILGALSYYIKRHKWAALK : 373
Cyt1p_D.melanogaster : ...DAPAGVVLREGQYFNPYFPGGAISMAQVLYNEVIEYEDGTPPTQSQLAKDVATFLKWTS.EPEHDDRKQLLIKVIGILGFLTVISYYIKRHKWSSLK : 296
Cyt1p_D.rerio : ...DPPAGVSLREGLYYNPYFPGQAIGMAPPIYNEVLEYDDGTP......CHHEPGWLRCLY.FPEVGSRART..... : 235
Cyt1p_X.laevis : ...DPPAGVTLREGLYYNPYFPGQAVGMAPPIYNEVLEYEDGTPATMSQVAKDVSTFLRWAS.EPEHDHRKRMGLKVLMISSILIPLIYYMKRHRWSVLK : 299
Cyt1p_M.Musculus : ...EPPTGVSLREGLYFNPYFPGQAIGMAPPIYTEVLEYDDGTPATMSQVAKDVATFLRWAS.EPEHDHRKRMGLKMLLMMGLLLPLTYAMKRHKWSVLK : 315
Cyt1p_H.sapiens : ...EPPTGVSLREGLYFNPYFPGQAIAMAPPIYTDVLEFDDGTPATMSQIAKDVCTFLRWAS.EPEHDHRKRMGLKMLMMMALLVPLVYTIKRHKWSVLK : 315
Cyt1p_E.gracilis : ..RPVPGGVQLSTTQWYNPYFHGGIIGMPPPLTDDMIEYEDGTPASVPQMAKDVTCFLEWCS.NPWWDERKLLGYKTIATLAVIAVSSGYYNRFLSGLWR : 230
Cyt1p_T.brucei : LMEIPPFAPELKPGQFWNPYFKDCVLSMPPPLSDGMVDYEDGTPATISQMAKDVVNFLRWSA.ESEYDDRRVMFWKCFTTLGLVNCILLHYCQKNTNWRI : 245

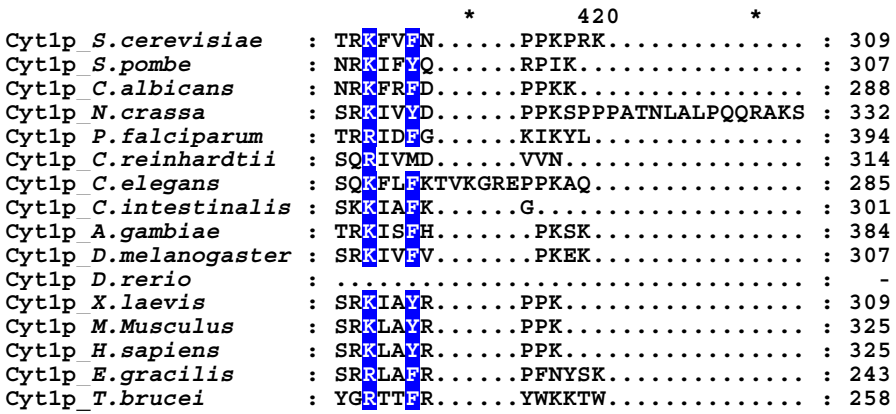

Figure 2 Supplemental data: Alignments of mitochondrial cytochrome c_1

Sequences of cytochrome c_1 (Cyt1p) from *Saccharomyces cerevisiae* (NP_014708), *Schizosaccharomyces pombe* (CAA18395), [1]*Candida albicans* (CA0864), *Neurospora crassa* (P07142), *Plasmodium falciparum* (AAN37210), *Chlamydomonas reinhardtii* (AJ417788), *Caenorhabditis elegans* (CAA99820), [2]*Ciona intestinalis* (ci0100131245), *Anopheles gambiae* (EAA04119), *Drosophila melanogaster* (AAM11126), *Danio rerio* (CA470874), *Xenopus laevis* (AAH45127), *Mus musculus* (NP_079843), *Homo sapiens* (NP_001907), *Euglena gracilis* (P20114) and *Trypanosoma brucei* (AAD19902) were aligned using CLUSTALW algorithm (Blosum62 scoring matrix) in Bioedit. The alignment was edited using the GeneDoc multiple alignment editor. Amino-acids strictly conserved in all sequences are shaded red and those conserved in the majority of the sequences (8 of 16) are shaded blue. The cysteine and histidine residues of the heme binding motif (CXXCH or CH) are highlighted in yellow. The position of the residues mutated in the suppressor alleles *CYT1-14*, *CYT1-34* and *CYT1-48* is highlighted in green and indicated by an arrow.

[1] See *Candida albicans* database at http://genolist.pasteur.fr/CandidaDB/index.html
[2] See *Ciona intestinalis* draft genome sequence at http://fugu.jgi-psf.org/ciona4/ciona4.home.html