Heme attachment to the apoforms of fungal mitochondrial cytochrome c and c₁ requires the activity of cytochrome c and c₁ heme lyases (CCHL and CC₁HL), 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 c₁, i.e. CCHL promotes low levels of apocytochrome c₁ conversion to its holoform in the absence of CC₁HL. 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 CC₁HL. Multicopy and genetic suppressors of the absence of CC₁HL were isolated and their analysis revealed that the activity of CCHL toward cytochrome c₁ can be enhanced by: 1) reducing the abundance of the cognate substrate apocytochrome c₁, 2) increasing the accumulation of CCHL, 3) modifying the substrate-enzyme interaction through point mutations in CCHL or cytochrome c₁, or 4) over-expressing 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 CXXCH 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-transducing membrane and maintained in a reduced form. The catalysis of 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 sulphydryls 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) and the cytochrome c₁ heme lyase (CC₁HL), for the two c-type cytochromes found in the mitochondrial intermembrane space, cytochrome c and cytochrome c₁, 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 c₁ (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 CC₁HL are related in sequence (35% amino acid identity) and display, with the notable exception of Plasmodium falciparum and Chlamydomonas reinhardtii CC₁HL, one to four typical heme regulatory motifs (see Fig. 1 of Supplemental Materials).

The abbreviations used are: CCHL, cytochrome c heme lyase; CC₁HL, cytochrome c₁ heme lyase; HCCS, holocytochrome c synthetase; LDS, lithium dodecyl sulfate; ORF, open reading frame; BLAST, basic local alignment search tool; DTT, dithiothreitol.

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that are present also in several other heme-binding proteins such as the transcription factor HapI (34) and heme oxygenase that are present also in several other heme-binding proteins. Ultimate biochemical characterization of CCHLs has not been thoroughly detailed. Ultimate biochemical characterization of CCHLs has not been thoroughly detailed. Sherman on the mechanism of the heme lyase reaction, including the in organello reconstitution of heme linkage to apocytochrome c by catalyzing thioether bond formation. Despite that the assembly factors mediate the formation of holocytochromes cchl active in the assembly of cytochrome cchl heme lyases in the formation of holocytochromes.

It is also not known whether other accessory factors assist the heme lyase activity of the purified proteins (28, 41, 42). cyc2 is conserved in yeast genomes and is likely to be conserved in the mitochrondrial enzymes (38). 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 c 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 has not been elucidated (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 cchl is only active in the assembly of cytochrome c1, CCHL in addition to its well established function in the biogenesis of holocytochrome c is also able to participate in the conversion of apocytochrome c1 to its holoform. We also demonstrate by functional complementation of the yeast cchl and cc1h1 mutants that the single heme lyase in human and mouse exhibits both the CCHL and CC1HL activities. Through multiplicity and genetic suppressor analyses, we revealed different mechanisms by which CCHL specificity is modified in favor of its non-cognate substrate apocytochrome c1. We also provide evidence that Cyc2p interacts functionally with CCHL in the assembly of holocytochrome c1 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 Δcycl, Δcyc2, Δcchl, Δcyc7, Δcy1, Δcc1h, or Δimp2 in the figures. The suppressed strains obtained spontaneously (R) or after UV-induced mutagenesis (UVx) are designated Δcchl sux when the suppressor allele is recessive and Δcchl SUX when the suppressor allele is dominant. Null alleles of yeast genes in the W303 background were constructed using a PCR-based method. The hphMX1 (46), loxP-kanMX-loxP (47), and HIS3MX8 (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 multiplicity wild type genome library constructed in the URA3-based pFL44L vector (a generous gift from F. Lagrouce) was used to search for suppressor genes able to alleviate the respiratory deficiency of the Δcchl and Δcc1h mutants. Transformants were plated on medium lacking uracil and replicated on glycerol medium to select for respiratory proficiency. Plasmids retrieved through the multiplicity 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/ or lactate as respiratory substrates.

Genetic Methods—Spontaneous or UV-induced respiratory competent revertants were selected from the SMY1 strain (Δcc1h). 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 ~10^8 revertants were recovered from 2 x 10^9 irradiated cells. No spontaneous (~10^-3) or UV-induced revertants (<2 x 10^-5) could be isolated from the SMY4 strain (Δcchl). 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 multiplicity 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-CCCHL Sc and pFL61-CC-HL Sc were constructed in a
similar way by cloning the ORFs of the yeast CYC3 and CYT2 genes, respectively, in pFL4L. The plasmids pFL4L-CYLH, pFL4L-CCHL, and pFL4L-CYT2 were generated as follows: a 1.3-kb SspI-Sall fragment containing the entire CCHL-encoding gene was cut from plasmid pRS316-CYC3 (a gift from R. Lill) and cloned into the PvuI-Sall sites of pFL4L; the CCHL encoding gene was cloned into pFL4L as a 1.7-kb PCR fragment using primers with engineered SacI and SalI sites and the CYC2 was cloned into pFL4L as a 1-kb SacI-SphI fragment from plasmid pGEM4-CYT2 (58). CCY2 was cloned into pFL4L as a 1.7-kb PCR fragment using primers with engineered SacI and SalI sites and the CYC2-harboring plasmid retrieved from the multicopySuppressor 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-32PdCTP 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′-GACTGAATTCAAGGCCGGTTCTC-3′ and 5′-GATATCGCAAAAAAGGGG-3′) and a 355-bp PCR product amplified with CYC7-specific oligonucleotides (5′-CAAGAAGATCATCACAAGC-3′ and 5′-GCAAACGGCTCTGTGACAGC-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 × 106 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).

Results
Conversion of Apo to Holocytochrome c1 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 and CCY1, and CCY2 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 c1 are both essential for electron transfer, the absence of either heme lyase was predicted to result in complete loss of respiratory growth. Surprisingly, we

3 We also observed a slow respiratory growth phenotype with the YS10 strain carrying a disrupted allele of the CCY1 gene (60).

4 For simplification, the CYC3 gene specifying CCHL and the CYT2 gene encoding CCY1, will be referred to as CCHL and CCY1, respectively, throughout the manuscript.

found that whereas the absence of CCHL leads to a tight respiratory deficiency, the absence of CCY1 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 phenotype5 of the Δccy1Δcyt1 strain segregated along with the null-allele in a genetic cross and was compensated upon expression of the CCY1 encoding gene (Fig. 3A). It is likely that the low respiratory activity of the Δccy1Δcyt1 mutant is attributable to the formation of holocytochrome c1 by the weak activity of the non-cognate CCHL.

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 c1 (Fig. 2, A and B), confirming previous work that holocytochrome c1 is synthesized in a separate pathway (31). No cytochromes aa3 were spectrally detectable in the Δcchl 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 Δccy1Δcyt1 mutant, the abundance of holocytochrome c1 could not be monitored by whole cell spectral analysis because of the increased level of holocytochrome c (see Fig. 2A and also Ref. 30) that masked the absorption peak of holocytochrome c1. 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 c1 was synthesized in the ccy1Δcyt1 mutant (Fig. 2C). The finding that this residual amount of holocytochrome c1 was no longer observed in the absence of both heme lyases indicates that CCHL displays some activity in the maturation of holocytochrome c1 (Fig. 2C). Immunoblotting analyses using an anti-cytochrome c1 antibody showed that the intermediate form of apocytochrome c1, which is the substrate for the heme attachment reaction, accumulated in the Δccy1Δcyt1 ΔccylΔcyt1 mutants (data not shown). As a control, we used a null-mutant in the IMP2 gene that encodes the protease re-
Absorption maxima of the 12.5% LDS-polyacrylamide gel and transferred to nitrocellulose membrane. Detection of holocytochrome 552 nm) detected in the cells grown at 28 °C on galactose medium was treated with sodium carbonate. Under these conditions, soluble holocytochrome whereas membrane-bound holocytochrome c spectra of cells grown in galactose were recorded with a Cary 400 spectrophotometer as described by Claisse et al.

LDS-polyacrylamide gel at 4 °C immunodecoration with antisera against cytochrome c. Following electrophoresis, the gel was transferred to polyvinylidene difluoride membrane before heme staining by chemiluminescence and processing by Imp2p (38, 68, 69), the previous finding that heme attachment precedes proteolytic

cyt 1, then it might be possible that CC1HL 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 overexpression of CCHL or CC1HL from a multicopy expression vector could result in increased activity of each lyase for its non-cognate cytochrome substrate. As shown in Fig. 3A, overexpression of CCHL suppresses the respiratory deficiency of the Δcc,hl and Δcchl Δcc,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 CC1HL (see Table II). Indeed, the amount of spectrally detected holocytochrome c1 was not restored to wild type upon expres-
sion of CCHL in the Δcc,hl and Δechl Δcc,hl strains (Fig. 3B and data not shown). This partial restoration of holocytochrome c1 could account for the weak suppression of the respiratory growth deficiency and suggests that the abundance of cytochrome c1 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,hl-null mutant led to an increased amount of the CCHL protein (Fig. 3C). On the other hand, overexpression of CC1HL did not suppress the tight respiratory phenotype due to the absence of CCHL, suggesting that CC1HL cannot promote assembly of holocytochrome c in the absence of its cognate lyase CCHL (Fig. 3A).

**Table II**

| Strain       | Plasmid              | Duplication time |
|--------------|----------------------|------------------|
| W303–1A      |                      | 2 h 30           |
| Δcc,hl       | pFL61-CCCHL Sc       | 2 h 20           |
| Δcc,hl       | pFL61-CC, HL Sc      | 2 h 20           |
| Δechl Δcc,hl | pFL61-CCCHL Sc       | 6 h 15           |
| Δechl Δcc,hl | pFL61-CC, HL Sc      | 7 h 10           |
| Δechl       | pFL61-HCCS Hs        | 2 h 20           |
| Δcc,hl       | pFL61-HCCS Hs        | 2 h 45           |
| Δechl Δcc,hl | pFL61-HCCS Hs        | 2 h 45           |

**Human and Mouse HCCS Function in the Assembly of Cytochrome c and Cytochrome c1**—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 cc,hl-null mutant and failure to compensate for the lack of CC1HL (72). This led to the proposal that insertion of heme into apocytochrome c1 in human is catalyzed by an enzyme other than HCCS or one that is considerably divergent from CC1HL (72). However, the demonstration in this work that yeast CCHL harbors both CCHL and CC1HL activities and the fact that only one heme lyase is present in the genomes of multi-cellular 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. 3A, 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
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 l-arabinose recovery of respiratory proficiency (see Table II). Nevertheless, it is reasonable to assume that the level of holocytchrome \( c_h \) in the \( cc_{11B} \)-null mutant suppressed by overexpression of Cyc2p is above the 5% wild type level of holoprotein detected in the \( cc_{11B} \)-null mutant (Fig. 2) because we have established through this study that restoration of the respiratory growth of the \( cc_{11B} \)-null mutant results systematically in enhanced levels of holocytchrome \( c_h \) (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 holocytchrome \( c \) (data not shown). The finding that a \( cc_{11B} \)-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 \( c_h \) (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. 3C). This also strengthens the view that Cyc2p suppression operates by modulating the CCHL activity.

Recessive and Dominant Genetic Suppressors Compensate for the Absence of CCHL—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 \( cc_1 \) and compensate for the respiratory deficiency. We were unable to isolate any suppressors from the \( cc_{11B} \) mutant but spontaneous and UV-induced suppressors of the absence of CCHL 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 \( c_h \) and spectral analysis on a subset of suppressed strains showed that recessive and dominant suppressor mutations resulted in partial restoration of
holocytochrome $c_1$ compared with the residual level detected in the $\Delta cc, hl$ 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

| Suppressor | Genetic nature | Respiratory growth$^a$ | Remarkable trait$^b$ | Molecular identification$^c$ |
|------------|----------------|-----------------------|---------------------|-----------------------------|
| $\Delta cc, hl$ su2 | Recessive | +/- | $\theta^a$ | CCHL |
| $\Delta cc, hl$ su19 | Recessive | + | $\theta^a$ | Cytochrome $c$ |
| $\Delta cc, hl$ SU9 | Dominant | ++ | $\theta^b$ | CCHL |
| $\Delta cc, hl$ SU14 | Dominant | ++ | $\theta^b$ | Cytochrome $c_1$, CA$_{im}^{å}$CH $\rightarrow$ CAPCH |
| $\Delta cc, hl$ SU34 | Dominant | ++ | $\theta^b$ | Cytochrome $c_1$, CA$_{im}^{å}$CH $\rightarrow$ CACCH |
| $\Delta cc, hl$ SU48 | Dominant | ++ | $\theta^b$ | |

$^a$ Growth on glycerol solid medium. In this classification, WT and $\Delta cc, hl$ growth correspond to $+++$ and $+$, respectively. $\theta^a$ and $\theta^b$ indicate the thermosensitivity (S) or the thermoresistance (R) of strains grown on glycerol solid medium at 37 °C. WT strain is $\theta^a$ and $\Delta cc, hl$ is $\theta^b$.

$^b$ Down accumulation (↓) or up accumulation (↑) of CCHL or cytochrome $c_1$, as observed in Fig. 7.

$^c$ ND, not determined.
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 Δcc·hl su19 suppressor strain. Interestingly, the absence of CC·HL 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·hl-null allele (see Fig. 2A). We concluded from this experiment that the decrease of cytochrome c in the Δcc·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 Δcc·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 c1 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 c1 assembly resulted from enhanced specificity of the CCHL for its non-cognate substrate cytochrome c1. This could occur through altered specificity of the enzyme (CCHL) for its non-cognate substrate (cytochrome c1) 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 Δcc·hl SU9 suppressor with a Δcc·hl mutant. The observation that all the single mutant Δcc·hl spores that we examined displayed a suppressed phenotype (i.e., restored respiratory growth in the absence of CC·HL) led us to conclude that locus 1 is linked to the CCHL-encoding gene. We sequenced the entire CCHL gene in the Δcc·hl SU9 suppressor and confirmed the presence of two contiguous mutations (TTTCTA to GTAATA) 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 c1, on the basis that all the single mutant Δcc·hl spores from a cross between a Δcc·hl SU9 strain and a Δcyt1 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 c1 (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 (TAC) change, SU16, -18, -34, -43, and -50 corresponded to an alanine (GCC) to proline (CCC) substitution and SU17 and -48 led to an alanine (GCC) to aspartic (GAC) mutation. We concluded from the study of the dominant suppressors that mutations in the substrate (cytochrome c1) 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·HL, 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 c1, whereas the specificity of yeast CC·HL is indeed restricted only to cytochrome c1, (ii) yeast CCHL activity toward apocytochrome c1 is enhanced by increasing the expression of CCHL, decreasing the expression of cytochrome c, or through missense mutations in CCHL or cytochrome c1, 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·HL 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 c1 can be acted upon by a single heme lyase. First, we have observed that the deletion of the entire CC·HL gene does not lead to a complete block of the respiratory activity indicating that a functionally overlapping factor must display some CC·HL activity. Then, we have demonstrated that the cc·hl-null mutant synthesizes low levels of holocytochrome c1 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 c1. 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. 6. Reduced accumulation of CYC1 and CYC7 mRNAs in the Δcc·hl su19 suppressor. RNA was extracted from wild type (WT) (W303-1A), Δcc·hl (SMY1), Δcc·hl su19 (R19), Δcyt1 (YPH9-2A), and Δcyt7 (W303ΔCYC7), and analyzed as described under “Experimental Procedures” using a-[32P]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.
Single human and mouse HCCS carries both CCHL and CC1HL activities. This indicates that in multicellular eukaryotes cytochrome c and cytochrome c1 are presumably assembled via the activity of one heme lyase.

**Strict Specificity of the Yeast CC1HL**—The complete lack of cytochrome c in the yeast cchl-null mutant indicates that cytochrome c cannot be acted upon in vivo by CC1HL. One possibility is that CC1HL 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 CC1HL 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 c1 import pathway by fusing at its amino terminus the bipartite targeting sequence of apocytochrome c1 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 CC1HL because the proper recognition elements for apocytochrome c are normally lacking in CC1HL. 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 CCHL toward apocytochrome c1 is strict and cannot be altered in favor of its non-cognate substrate apocytochrome c. Nevertheless, it is possible that mutations can convert CC1HL into a CCHL but could not be revealed in our screen because of their deleterious effect on holocytochrome c1 assembly. Alternatively, more than one mutational event might be required for CC1HL to act upon apocytochrome c.

**Alterations of the Expression of CCHL or Cytochrome c Favor CCHL Activity Toward Cytochrome c1**—Increased accumulation of CCHL through multicopy or genetic suppression yields enhanced activity of CCHL toward its non-cognate substrate apocytochrome c1. Interestingly, we found that a modest increase (~2-fold) in the abundance of CCHL can suppress the absence of CC1HL in the su2 suppressor. This is consistent with the observation that suppression of the cc1hl-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 CC1HL. This slight increase may generate some excess CCHL that is not already bound to apocytochrome c and therefore can act on apocytochrome c1. This view is corroborated by the fact that reduced accumulation of the cytochrome c substrate in a cc1hl-null background leads to an increase in holocytochrome c1 accumulation. This suggests that, in the absence of CC1HL, holocytochrome c1 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 cc1hl 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 c1.

**Mutations in CCHL or Cytochrome c1 Increase CCHL Activity Toward Cytochrome c1**—The view that cytochrome c1 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 CC1HL. Because wild type CCHL has the intrinsic ability to function as a C1HL, it is conceivable that the suppressor mutations modify residues involved in the interaction between CCHL and apocytochrome c1 and improve affinity of the enzyme for the apocytochrome c1 substrate. Limited information is available regarding the sequence requirements for recognition of apocytochrome c and c1 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 c1 as a recognition site for CCHL in vivo. Mutation of the serine residue located 24 residues upstream of the CXCHX 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 c1, we hypothesize that this mutation modifies the interaction of CCHL to favor cytochrome c1. There is the possibility that other sites of interaction in CCHL and cytochrome c1 exist but could not be uncovered because our screen is based on restoration of respiratory activity that relies on both functional holocytochrome c and c1.

**Fig. 7. Accumulation of CCHL and cytochrome c in suppressor strains.** Strains used were: wild type (WT) (W303-1A), Δcc1hl (SMY1), Δcc1hl (SMY4), Δcc1hl su2 (B2), Δcc1hl su19 (B19), Δcc1hl SU34 (UV34), and Δcc1hl 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.
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 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 assembly in the absence of CC1HL reveals that the function of this protein is not limited to the biogenesis of holocytochrome c but also extends to that of holocytochrome c1. We hypothesized that Cyc2p suppresses the absence of CC1HL by modulating the activity of CC1HL in favor of its non-cognate substrate apocytochrome c1. The fact that we did not retrieve CHL as a multicyclic suppressor of Δcc1hl indicates that our screen for suppressors is not saturated and that other modulators of CHL activity could still be revealed. That Cyc2p interacts functionally with CC1HL and participates in the heme attachment reaction is inferred from our finding that a cyc2-null cc1hl-null mutant displays a synthetic respiratory block that can no longer be suppressed by overexpression of CHL or the suppressor mutations in CHL or cytochrome c1. Therefore, whereas the function performed by Cyc2p in cytochrome c biogenesis appears redundant in a wild type background, it becomes essential when CC1HL is inactive. The mechanism by which Cyc2p could control the activity of CHL 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 investigation in system III because of the belief that the mitochondrial intermembrane space was a reducing compartment. Yet, 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 c1 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 c1 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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