Genetic Analysis of Chloroplast c-Type Cytochrome Assembly in
Chlamydomonas reinhardtii: One Chloroplast Locus and at Least
Four Nuclear Loci Are Required for Heme Attachment

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ABSTRACT

Chloroplasts contain up to two c-type cytochromes, membrane-anchored cytochrome f and soluble cytochrome c₆. To elucidate the post-translational events required for their assembly, acetate-requiring mutants of Chlamydomonas reinhardtii that have combined deficiencies in both plastid-encoded cytochrome f and nucleic-encoded cytochrome c₆ have been identified and analyzed. For strains ct34 and ct59, where the phenotype displays uniparental inheritance, the mutations were localized to the chloroplast ccsA gene, which was shown previously to be required for heme attachment to chloroplast apocytochromes. The mutations in another eight strains were localized to the nuclear genome. Complementation tests of these strains plus three previously identified strains of the same phenotype (ac206, F18, and F2D8) indicate that the 11 ccs strains define four nuclear loci, CCS1–CCS4. We conclude that the products of the CCS1–CCS4 loci are not required for translocation or processing of the preproteins but, like CcsA, they are required for the heme attachment step during assembly of both holocytochrome f and holocytochrome c₆. The ccsA gene is transcribed in each of the nuclear mutants, but its protein product is absent in ccs1 mutants, and it appears to be degradation susceptible in ccs3 and ccs4 strains. We suggest that Ccs1 may be associated with CcsA in a multisubunit “holocytochrome c assembly complex,” and we hypothesize that the products of the other CCS loci may correspond to other subunits.

The c-type cytochromes, virtually ubiquitous in energy-transducing membranes, are distinguished from other heme proteins and cytochromes by the covalent attachment of the heme cofactor to the polypeptide at a conserved CxCH sequence near the amino terminus of the protein. Cytochrome c₅₅ and c in mitochondria and respiring bacteria function to oxidize quinols and reduce a terminal oxidase, while analogous chondria and respiring bacteria function to oxidize quinols and reduce a terminal oxidase, while analogous cytochromes in chloroplasts (cytochromes f and c₆) and photosynthetic bacteria function to oxidize quinols and reduce a photo-oxidized reaction center. In some photosynthetic bacteria, for example, Rhodobacter spp. or many cyanobacteria, some of the cytochrome components of the energy-transducing membrane are shared between the photosynthetic and respiratory electron transfer chains.

Because the c-type cytochromes are so well studied with respect to structure and function, they have also served as excellent models for the study of cofactor protein assembly in many experimental organisms. These studies have revealed three types of cytochrome maturation pathways: one occurring in fungal, mammalian, and nematode mitochondria (exemplified by Saccharomyces cerevisiae), a second occurring in plant mitochondria and most of the proteobacteria (exemplified by Rhodobacter spp. and rhizobia), and a third found in chloroplasts, the gram-positive bacteria and Helicobacter pylori.

For S. cerevisiae, extensive genetic analyses of respiration-defective strains revealed a number of loci that were required for cytochrome c and c₅₅ synthesis, accumulation, and function (Sherman and Stewart 1971; Lang and Kaudewitz 1982; Matner and Sherman 1982). Of these, only the CYC3 and CYT2 loci were shown to be essential for the heme attachment step to apocytochrome c and c₅₅, respectively, and it was suggested that these loci encoded cytochrome c and c₅₅ heme lyases (Dumont et al. 1987; Zollner et al. 1992). The biochemical function of these enzymes is deduced to lie in the catalysis of thioether bond formation, and each appears to be specific for its respective apoprotein substrate.

By contrast, genetic analyses of c-type cytochrome biogenesis in the gram-negative bacteria has revealed many genes whose products are required for heme at-

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attachment to a variety of apocytochromes, including membrane and soluble forms. The cytochrome c assembly-deficient mutants of bacteria generally exhibit a pleiotropic c-type cytochrome-minus phenotype, and therefore, also pleiotropic metabolic deficiencies. Genes required for c-type cytochrome biogenesis were cloned from Rhodobacter capsulatus (hd, cd loci), Bradyrhizobium japonicum (yc loci), and Paracoccus denitrificans by complementation of the mutant phenotypes (Kränz 1989; Ramseier et al. 1991; Beckman et al. 1992; Beckman and Kränz 1993; Page and Ferguson 1995; Ritz et al. 1995).

Chloroplasts contain up to two c-type cytochromes. Cytochrome f (cyt f), found in all chloroplasts and in cyanobacteria, is a membrane-associated subunit of the cytochrome b_{6}/f complex, and it is anchored to the membrane via a hydrophobic sequence near its C-terminal end (Wiley and Gray 1988; Kuras et al. 1995). A large, soluble N-terminal domain containing the heme group extends into the lumen, where it can interact with its substrate plastocyanin, or in some green algae, with cytochrome c_{6} (cyt c_{6}). Cyt c_{6} is a soluble, lumen-localized protein that substitutes for plastocyanin in copper-deficient cultures of various green algae and cyanobacteria (reviewed by Merchant 1997).

Previously, we identified a class of Chlamydomonas reinhardtii mutants that were deficient in both chloroplast c-type cytochromes but contained normal amounts of mitochondrial cytochromes and a photosystem II cytochrome (Howe and Merchant 1992). Because the two plastid c-type cytochromes, f and c_{6}, are encoded in different genomes, it seemed highly unlikely that the mutations might affect the expression of the plastid pdA and nuclear Cyc6 genes encoding the respective polypeptides; the normal abundance of mitochondrial cytochromes and photosystem II function argued against a defect in the cofactor biosynthetic pathway. It was rather more likely that a common posttranslational assembly step might be affected in these mutants. Indeed, pulse-chase analysis of cyt c_{6} synthesis and processing in wild-type vs. mutant strains revealed that (1) heme attachment occurred in the thylakoid lumen (Howe and Merchant 1994) and (2) the mutants were blocked at the step of heme attachment (Howe and Merchant 1992; Howe et al. 1995). Based on the identification of this class of mutants, we concluded that cyt f and cyt c_{6} assemble via a common pathway in the thylakoid lumen.

The recognition of this pleiotropic phenotype facilitated the identification of additional heme attachment mutants. In this article, we describe the genetic and biochemical analyses of these cytochrome assembly mutants. Five complementation groups are named: one group, defined by strains B6, ct34, and ct59, corresponds to the chloroplast ccsA gene, while the other four, CCS1-CCS4, represent nuclear loci. We suggest that multiple biochemical functions, encoded perhaps by the products of the CCS1-CCS4 loci, are required for handling the heme and apoprotein substrates of a chloroplast c-type cytochrome assembly complex.

MATERIALS AND METHODS

Strains and culture conditions: Chlamydomonas reinhardtii wild-type strain CC125 (MT+ ) was obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). Strains ac206 (now ccs1-ac206), F18 (now ccs1-F18), and F208 (now ccs4-F208) have been described previously (Gorman and Levine 1966; Lemaire et al. 1986; Howe and Merchant 1992). Ten new c-type cytochrome-deficient strains are described in this work. Eight (ccs1-2, 1-3, 1-4, cs2-1, 2-2, 2-3, 2-4, 2-5) were identified from a population of UV-mutagenized CC125 cells, and two (ct34 and ct59) were identified from a population of chemically mutagenized cells (see below). Each original mutant strain was crossed with a wild-type strain of the Paris collection (derived from 137c) to obtain mutant strains of both mating types for the genetic analyses. Cultures of wild-type strains were grown at 22–25°C in TAP medium (Har ris 1989) under cool fluorescent lights (15–125 μm - m^{-2} s^{-1}) with agitation (225 rpm). Mutant strains were grown under the same conditions, except that the illumination was always reduced (15–25 μm - m^{-2} s^{-1}).

Identification of candidate heme attachment mutants: CC125 cells were mutagenized by UV irradiation, subjected to metronidazole enrichment, and screened for phototrophic growth on minimal medium, as described previously (Li et al. 1996). Strains ct34 and ct59 were obtained after treatment with fluoro(deoxy)uridine and enrichment with metronidazole, as described by Bonneau et al. (1978), and were identified as cytochrome b_{6}/f-deficient on the basis of their fluorescence induction kinetics during a dark-to-light transition and the absence of characteristic polypeptides in thylakoid membrane preparations.

Cytochrome b_{6}/f-deficient strains were identified from the population of acetate-requiring mutants by testing for cyt f accumulation. The absence of cyt f implies the absence of the entire cytochrome b_{6}/f complex because cyt f accumulation is required for the accumulation of the other polypeptides of the cytochrome b_{6}/f complex (Kuras and Wollman 1994). Each candidate acetate-requiring strain was grown as a lawn on a TAP agar (1.5%) slab in a 100-mm petri dish. After 2–3 wk of growth in dim light, the cells were scraped off with a razor blade and resuspended in a minimal volume (≈50 μl) of 10 mm sodium phosphate (pH 7.0). The cells were lysed by slow freeze-thaw and separated by centrifugation into soluble and membrane protein fractions (supernatants vs. pellets). The pellet fractions were resuspended in 75–100 μl of a solution containing 10 mm sodium phosphate (pH 7.0), 1 mm phenyl methyl sulfonyl fluoride, 5 mm e-aminoacaproic acid, and 1 mm benzamidine, and were either analyzed immediately or stored frozen for future analysis. Before analysis, pellet fractions equivalent to 6 μl of chlorophyll were collected by centrifugation, resuspended in 100 μl of TrisCl buffer (62.5 mm TrisCl (pH 6.8), 2% sodium dodecyl sulfate, 20% glycerol, 5% 2-mercaptoethanol plus protease inhibitors as described above), and heated to 90°C for 10 min followed by centrifugation (3 min, 12,000 g) to remove insoluble debris. The solubilized proteins were tested for cyt f content after electrophoresis (SDS-containing, 12% acrylamide gels) and transfer (to Immobilon P; Millipore, Bedford, MA) by immunoblot analysis (1:500 dilution of an antispinach cyt f antiserum). Strains that displayed a cyt f deficiency were transferred to liquid medium and restested to confirm the deficiency. Confirmed cyt f-deficient mutants were also screened for the accumulation of the following proteins: ATP synthase, the OEE1 protein of PSI, and cyt c_{6}. For
the initial screen for the cytochrome c₆ deficiency candidate strains were grown on copper-deficient TAP agar (1.5%) slabs (Quinn and Merchant 1995), and soluble extracts were tested for cyt c₆ and plastocyanin abundance (Li et al. 1996). Subsequent to the primary screen, strains were cultured in copper-deficient liquid medium for further analyses of cyt c₆. All strains were tested routinely for their fluorescence properties (see above) to confirm that they displayed a characteristic cytochrome b₆/f-deficient phenotype. This is essential, because the complementation tests rely on the fluorescence phenotype. Furthermore, the accumulation of suppressors can be avoided.

Genetic analysis of cytochrome-deficient nuclear mutants: Genetic analyses were carried out as described previously (Harris 1989; Goldschmidt-Clermont et al. 1990). For the tight linkage tests, at least 30 zygotes were transferred the same day to either TAP or minimal agar plates, and they were separated from each other along a line. Zygotes giving rise to colonies on the TAP plate were counted after 2 wk of incubation under low light; this gives an estimate of the viability of the zygotes and their progeny. Zygotes that gave rise to colonies on the minimal plate were counted after ~4 wk of incubation under high light: this gives an estimate of zygotes giving rise to wild-type progeny (TT and NPD tetras). For complementation tests, ~1.5 ml of mixed gametes were transferred to small petri dishes (35 × 10 mm) and incubated under high light overnight. The petri dish was transferred to low light after the zygotes aggregated and formed a continuous pellicle. After 3 days, the zygote pellicle was washed with H₂O to eliminate unmated gametes. Fluorescence measurements to reveal the presence or absence of cytochrome b₆/f function were performed directly on the zygote pellicle in the petri dish.

Protein preparation and analysis: Freeze-thaw fractionation and analysis of supernatant and pellet fractions by electrophoresis and immunodetection have been described previously (Howe and Merchant 1992; Li et al. 1996). For detection of CcsA, the transfer (50 V, 2.5 hr) was performed in the presence of SDS (0.01%) and without methanol. The ccsA gene product was misidentified in previous work as a 29-kD protein in the soluble fraction of cells (Xie and Merchant 1996). More recent studies indicate that the protein fractionates with the thylakoid membrane and has a relative mobility corresponding to a molecular weight slightly greater than 33 kD (B. W. Dreyfuss and S. Merchant, unpublished results). The transfer conditions are critical for quantitative transfer of the ccsA gene product. For optimal detection of CcsA, the samples must be prepared fresh.

The primary antisera (described in Howe and Merchant 1992, 1993; Xie et al. 1995; Xie and Merchant 1996) were diluted as follows: antiplastocyanin (1:5000), anti-cyt c₆ (1:1000), anti-cyt f (1:500), anti-OEE1 (1:1000), and anti-CcsA (1:500).

Bound primary antibody was detected with an alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody according to the instructions provided by the manufacturer (Bio-Rad, Richmond, CA). For detection of heme proteins, the membranes were washed in Trisbuffered saline, incubated with the reagents for the chemiluminescence assay (Persigual; Pierce Chemical Co., Rockford, IL), and immediately exposed to Medical RX film (Fuji Photo Film Company, Tokyo, Japan).

Analysis of the ccsA gene in the uniparental mutants: Rescue of the mutant phenotype of strains ct34 and ct59 with the cloned wild-type ccsA gene was performed exactly as described previously (Xie and Merchant 1996). The NdeI-HindIII fragment containing the entire ccsA coding region (and corresponding to the minimal complementing fragment) was cloned from strains ct34 and ct59 according to the methods described previously (Xie and Merchant 1996). The fragment was sequenced completely on both strands at the UCLA DNA-sequencing facility. Any difference noted between the cloned wild-type ccsA sequence and that of the ccsA genes of ct34 and ct59 was confirmed by sequencing an independent amplification product.

RNA preparation and analysis: The procedure for RNA isolation and RNA blot analysis has been described previously (Merchant and Bogorad 1986; Hill et al. 1991). The abundance of the ccsA RNA in strains ct34 and ct59 was estimated by amplification of a cDNA, as done in a previous work (Xie and Merchant 1996). For amplification of the ccsA RNA from the ccsA-ccs4 strains, the procedure was modified to ensure that the conditions were suitable for quantitative estimation of transcript abundance. Specifically, a random primer pdN₉ (Pharmacia Biotech, Piscataway, NJ) was used (2.5 nmol/40 μl reaction) instead of primer 15-1 for reverse transcription, the reverse transcription product was used for amplification (with primers 15-1 and 15-2) without further purification, and the cycle conditions (on a GeneAmp PCR system 2400; Perkin Elmer, Norwalk, CT) were as follows: one cycle at 95° for 5 min, 25 cycles at 95° for 30 sec, 54° for 30 sec, and 72° for 30 sec, followed by a final 5-10 min extension at 72°. The abundance of the pdA transcript was estimated simultaneously by amplification with primers cyt-f-1 (5'-TTACCACTGGTATGGCGG-3') and cyt-f-5 (5'-AACGAGCTTCCCTTATAG-3'). The yield of both products was dependent on the amount of input RNA (from 0.25 to 2 μg) and input cDNA (from 1 to 10 μl of reverse transcription product). The amount of pdN₉ primer was determined to be saturating for synthesis of the cDNA, and the subsequent amplification reaction was in the exponential stage up to 30 cycles.

Pulse-radiolabeling and immunoprecipitation: To monitor cyt c₆ synthesis, cells were grown in copper-deficient, reduced-sulfate medium, washed in TAP medium lacking trace elements and sulfate, and maintained in that medium throughout the period of labeling and “chase,” while for cyt f synthesis, the cells were grown and maintained in the usual copper-supplemented medium. The labeling was conducted at 22°, as described previously (Li et al. 1996). After 10 min of labeling, a “chase” was initiated by the addition of unlabeled sulfate (24 mm) and cycloheximide (46 μg/ml) for studies of cyt c₆, or chloramphenicol (250 μg/ml) for studies of cyt f. The cells were sampled into acetone, and the protein of interest was immunoprecipitated from the resolubilized, dried pellets. The effectiveness of the labeling and chase was assessed by measuring TCA-insoluble radioactivity in the resolubilized acetone pellet and, occasionally, by monitoring the labeling of plastocyanin (whose accumulation is unaffected in these mutants).

For immunoprecipitation of cyt f, 95 μl of the resolubilized acetone pellet from 200 μl of labeled cells was diluted with 0.4 ml of a 5% solution of IgGSOB in immunoprecipitation buffer [40 mm TrisCl (pH 7.5), 150 mm NaCl, 2 mm EDTA, 2% Nonidet P-40], and the sample was agitated at room temperature (on a rocker) for at least 30 min. The IgGSOB plus nonspecifically bound proteins were removed by centrifugation (12,000 g, 5 min). Aprotinin (10 μl of 1 mg/ml stock) and antiserum (10 μl of anti-C. reinhardtii cyt f; Chen et al. 1995) were added to the supernatant, and the mixture was left overnight at 4°. The antigen-antibody complex was collected by treatment with 50 μl of a 20% IgGSOB solution. After removal of the antigen-antibody complex with IgGSOB, the supernatant was treated with anti-cyt f to assess the effectiveness of the immunoprecipitation. The IgGSOB-bound antibody-antigen complex was washed three times with 0.7 ml of immunoprecipitation buffer and once with 0.9% NaCl, and was then released from the IgGSOB into 0.1 ml of solution A by heating at 95° for 5 min (Li et al. 1996). The IgGSOB was removed by centrifugation, and the supernatant containing
the antigen was diluted with 4 volumes of immunoprecipitation buffer and subjected to a second round of immunoprecipitation to reduce the background. The final immunoprecipitate was analyzed by denaturing gel electrophoresis followed by fluorography (Li et al. 1996). For extracts of mutant strains that do not accumulate cyt f, the immunoprecipitation was essentially quantitative for newly synthesized cyt f. In the case of wild-type extracts, two sequential treatments with anti-cyt f were necessary to “pull down” all the cross-reacting material. For quantitation of cyt f synthesis, the signal from both immunoprecipitates was added and expressed relative to total incorporation of 35S into acid-insoluble material.

Cyt c6 was immunoprecipitated as described previously (Howe and Merchant 1992; Li et al. 1996), except that the samples were not subject to any preabsorption. Antisera (20 μl) was used for acetone precipitates corresponding to 125 μl of labeled cells. A fraction (20–40%) of the final immunoprecipitate was analyzed by electrophoresis (12% acrylamide) in the system described by Giulian et al. (1983). Cyt c6 was immunoprecipitated from labeled wild-type cells (10 min of radiolabeling followed by 10 min of incubation with unlabelled sulfate) to generate a holocytochrome c6 standard sample, and an apocytochrome c6 standard was generated by immunoprecipitation of anti-cyt c6-reactive species from labeled B6 cells (10 min of radiolabeling). A small fraction (5–10%) of the solubilized immunoprecipitate was used in each track.

RESULTS

Identification of cyt f / cyt c6 mutant strains: A collection of metronidazole-enriched, nonphotosynthetic strains generated by either chemical (Bennoun et al. 1978) or UV mutagenesis (Li et al. 1996) was screened for deficiencies in the cytochrome b6/f complex. The chemically mutagenized strains were analyzed by screening dark-adapted colonies for their fluorescence induction and decay kinetics upon illumination. Mutants with defects in the cytochrome b6/f complex are blocked in the electron flow beyond the plastoquinol pool, and this results in a characteristic fluorescence signature (Bennoun and Delpeule 1982). Candidate cytochrome b6/f-deficient strains were analyzed by examination of thylakoid membrane protein profiles after denaturing gel electrophoresis, and 13 strains that appeared to lack the major subunits of the cytochrome b6/f complex were chosen for further study. For the acetate-requiring strains resulting from UV mutagenesis and metronidazole enrichment, cytochrome b6/f deficiencies were identified by the estimation of anti-cyt f-reactive polyproteins in the mutant strains in comparison to the wild type. One hundred eighty-eight strains were chosen from a preliminary screen of 1035 acetate-requiring strains. Thus, the collection of cytochrome b6/f-deficient strains totalled 201.

In previous work, we showed that among the cytochrome b6/f-deficient mutant strains, a subgroup that lacked cyt c6 was identified, and this subgroup represented those mutants with defects in the process of heme attachment (Howe and Merchant 1992). Each mutant strain was therefore transferred to copper-deficient medium (to induce Cyc6 expression), and soluble extracts were analyzed for cyt c6 accumulation. Twelve of the 201 cytochrome b6/f-deficient strains displayed severe defects in cyt c6 accumulation. To confirm that the cells were tested under Cyc6-inducing conditions, extracts were assayed in parallel for plastocyanin abundance (not shown). Indeed, the absence of plastocyanin confirmed that the medium was sufficiently copper-deficient to allow expression of the Cyc6 gene.

To assess the severity of the cytochrome deficiencies more quantitatively, the soluble and membrane proteins of each mutant strain were analyzed in parallel with the dilutions of wild-type extracts. An example of one experiment where 11 mutant strains were analyzed in parallel for plastocyanin, OEE1, and c-type cytochrome abundance is shown in Figure 1. The heme stain assay measures the accumulation of the assembled holocytochromes (Figure 1, A and B), while the immunoblots (Figure 1, C and D) measure the abundance of the cyt f and cyt c6 polypeptides (apo- and holoproteins). From a number of experiments wherein the original mutant strains or spores derived from the backcrosses were analyzed over a period of 4 yr, we concluded that both holocytochromes c6 and f were present generally at <5% of wild-type levels in the mutant strains (see Figure 1, A and B). While strains ccs1-3, ccs1-4, ccs2-1, and ccs2-3 display a stringent nonphotosynthetic phenotype, strains ccs1-2, 2-2, 2-4, and 2-5 exhibit a leaky acetate-requiring phenotype and grow slightly on minimal medium. For these “leaky” mutants, the abundance of cyt f and cyt c6 was noted occasionally to be as high as 10% of wild-type levels. Each mutant was backcrossed to the wild type, and between 6 and 20 tetrads were tested for their fluorescence kinetics. In each tetrad of crosses involving either stringent or leaky mutants, a 2:2 segregation of the original phenotype was observed. The immunoblot signal is not always well-correlated with the heme stain (e.g., Figure 1, lane 1, A vs. C). This may reflect the fact that the abundance of the apoprotein depends on the absolute rate of apocytochrome f synthesis and degradation, and we have certainly seen strain-specific variations in the rate of degradation of apocytochromes (τ1/2 ~10–30 min; see Figure 3, for example). The extracts were also tested for the abundance of other thylakoid membrane proteins to assess the specificity of the phenotype. Indeed, the deficiencies appeared to be restricted to the cytochrome b6/f complex and cyt c6. For instance, immunoblot analysis of plastocyanin (Figure 1E), OEE1 (Figure 1F), and the subunits of the ATP synthase (not shown) indicated that these proteins were found at normal levels in each strain. Denaturing electrophoretic analysis of thylakoid membranes prepared from the mutant strains revealed no other obvious deficiencies, and fluorescence kinetics during a dark-to-light transition indicated normal PSII function in each mutant.

Finally, RNA blot analysis indicated that accumulation of the petA, B, and D transcripts, encoding cyt f,
cyt b₆ and subunit IV, respectively, was normal in each mutant strain (data not shown). Likewise, the Cyc6 gene was induced appropriately in copper-deficient cells of each mutant strain. The initial characterization of this class of mutants indicated that they were phenotypically identical to the previously defined heme attachment mutants B6, ac206, F18, and F2D8 (Gorman and Levine 1966; Lemaire et al. 1986; Shochat et al. 1990; Howe and Merchant 1992).

**Genetic analyses of the ccs mutants:** Backcrosses of the mutant strains (named ccs for c-type cytochrome synthesis) indicated that two strains, ct34 and ct59, displayed uniparental inheritance while the rest displayed Mendelian inheritance. Recombination tests between ct34 and B6 failed to yield recombinants (L. Mets, personal communication), which suggested that they may represent alleles. Since the B6 mutant results from a frame-shift mutation in the plastid-encoded ccsA gene (Xie and Merchant 1996), strains ct34 and ct59 were tested for ccsA expression by estimating the abundance of ccsA transcripts and the CcsA polypeptide. Although ccsA transcripts are present at levels comparable to wild type (Figure 2A), the protein product cannot be detected (Figure 2B). This suggested that the mutation in strains ct34 and ct59 might indeed be localized to the ccsA gene, and this was confirmed by complementing the strains with the cloned wild-type ccsA gene (data not shown).

The ccsA gene, encoding a 353-residue hydrophobic protein, was amplified from strains ct34 and ct59 and was sequenced to identify the nature of the mutations (Figure 2C). Strain ct34 was found, like strain B6, to carry a single nucleotide deletion (deletion of one T from a string of seven Ts) at the 23rd codon of the open reading frame defined by the first ATG, and strain ct59 was found to carry a similar mutation (deletion of one T from a string of six Ts) at the 269th codon (data not shown; 100% of the sequence was determined on both strands). The position of the mutation in strain ct59 emphasizes the importance of the C-terminal end of the protein with respect to structure. Although three-quarters of the protein (residues 1–268 out of 353) must be translated normally, the strain does not accumulate a truncated version of the protein (data not shown), which suggests that deletion of the C-terminal 24% of CcsA (residues 269–353) must render it protease susceptible. Accordingly, the phenotype is just as severe as that of strain B6, which carries a mutation at the 23rd codon.

In addition to the 10 new nuclear mutants identified in this work, another three candidate heme attachment mutants were available from previous work (strains F18, F2D8, and ac206). Although strain ac206 had been mapped to linkage group XIV (Harris 1989), the ccs class of mutants had not been subjected to thorough genetic analysis. To assess the number of genes represented by these strains, complementation tests were
A

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B

WT

B6

c34

c59

C

WT

B6 & ct34

c59

Figure 2.—Expression of ccsA in strains ct34 and ct59. (A) Total RNA was isolated from wild-type and mutant strains, digested with RQ1 DNase (Promega, Madison, WI), and used as a template for reverse transcription (5 μg per reaction). The cDNA was detected by amplification with ccsA-specific primers (lanes marked +RTase). No products were observed when the amplification reaction was performed on the same RNA preparation but without reverse transcription (lanes marked −RTase). The plasmid pEBH (Xie and Merchant 1996) containing the cloned ccsA gene was used as a template for amplification with the same primers to generate a standard product (lane marked plasmid). (B) Pellet fractions (corresponding to 20 μg chlorophyll) of protein preparations from wild-type (WT) or mutant strains were tested for the presence of the CcsA polypeptide by immunoblot analysis. The solubilized proteins were separated by electrophoresis (60 V, 18 hr) on an SDS-containing acrylamide (12%) gel (16 cm, lower gel), transferred to polyvinylidifluoride membranes (50 V, 2.5 hr), incubated overnight with the anti-CcsA antiserum, and an alkaline-phosphatase-conjugated secondary antibody was used to detect the bound primary antibody. The blot was developed for 10 min with a chromogenic substrate. (C) The DNA sequence of the ccsA gene of mutant strains B6, ct34, and ct59, as well as the corresponding deduced amino acid sequence in the vicinity of the mutation in each strain is shown. The position of the single nucleotide (T) deletion in each case is indicated by a white character on a black background, and the resulting premature termination codon is shaded. The subscripts numbers refer to the last amino acid encoded in the mutant ccsA genes.

undertaken via pairwise crosses (Table 1). Complementation can be assessed by testing the fluorescence induction kinetics of the resulting diploid zygotes (Bennoun et al. 1980; Goldschmidt-Clermont et al. 1990). Zygotes with mutations that complement each other exhibit fluorescence curves with a decay phase resembling that displayed by the wild type. Zygotes of strains carrying two noncomplementing mutations exhibit fluorescence curves without a decay phase as do the parental mutants. The complementation tests were supplemented by tests for tight linkage from the same sets of crosses. Mutations in the same gene should be very tightly linked, and wild-type recombinants would not be expected for crosses between strains carrying mutations in the same gene. The results of the complementation and tight linkage tests are presented in Table 1. Accordingly, the consistency between the results from the two tests lead us to classify 11 of the 13 mutations studied to four nuclear genes that correspond to the CCS1–CCS4 loci. The possibility of dominant alleles that might also fail to complement can be ruled out because all mutations do complement members of other groups. Thus, we deduce indirectly that the mutations are recessive. For each mutation, homozygotes were also tested in minimal medium to (1) have a measure of the growth phenotype for each mutant strain and (2) estimate the potential for “reversion.” The results are indicated on the diagonal in Table 1.

Synthesis of apocytochrome f and apocytochrome c₆ in the ccs mutants: To confirm that the mutants were able to synthesize cyt f and cyt c₆, synthesis was assessed in pulse-radiolabeled cells. Synthesis of cyt f appeared to be normal in the ccs mutants during a 10-min labeling period (representative examples in Figure 3, lanes P). For example, for strain ccs3-f18, the amount of label incorporated into cyt f during the 10-min labeling period was ~60% of that for a wild-type strain (see materials and methods and figure legends for description of quantitation). The electrophoretic mobility of the immunoprecipitated material from each ccs strain indicated that it represented the mature, processed apoprotein. Thus, synthesis and processing of cyt f occurs normally in the ccs mutants; however, the newly synthesized protein is short-lived in the mutant strains compared to the wild type (Figure 3, lanes C). The short half-life (which varies between 10 and 30 min for individual strains) accounts for the differential accumulation of cyt f in the ccs mutants. The disappearance of the newly synthesized protein is attributed to the degradation of the unassembled subunit rather than to cell lysis or nonspecific protein degradation during the course of the experiment because other newly synthesized proteins (e.g., plastocyanin) are stable during the chase (not shown).

A recent study of the cyhl locus in Rhodobacter capsulatus showed that certain cyhl alleles displayed differential effects on the maturation of membrane vs. soluble cytochromes (Lang et al. 1996). Thus, each mutant strain
Complementation analysis of cyt f⁻/ cyt c₆⁻ strains reveals four nuclear loci

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The results of the complementation test are shown above the diagonal (top and right). The fluorescence induction kinetics of sheets of zygotes during a dark-to-light transition were scored 2–4 days after mating. The efficiency of mating was ~80–90%. A plus sign indicates that the zygotes displayed curves corresponding to a phenotype of wild-type zygotes, i.e., positive complementation, while a negative sign indicates that the zygotes displayed curves corresponding to a mutant phenotype, i.e., the two strains failed to complement. The results of the tests for tight linkage are shown below the diagonal (bottom and left). The growth of progeny of zygotes was assessed on minimal medium vs. TAP medium. The scores in the table represent the number of zygotes that germinated and gave rise to colonies on minimal medium, a1 is the number of zygotes transferred to TAP medium, and b1 is the number of zygotes that gave rise to colonies on TAP medium, and b2 is the number of zygotes transferred to TAP medium. For crosses that were repeated with different isolates of the mutant strains, the results are shown separately.

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The results of reversion tests are shown on the diagonal. The growth of zygote progeny in crosses with leaky mutants could be attributed to reversion of the strains rather than to recombination.

studied in this work was characterized with respect to cyt f and cyt c₆ synthesis. Pulse-radiolabeling studies indicated that cyt c₆ is synthesized normally in each of the mutant strains, and that post-translational processing of the precursor and intermediate form to the mature form occurs at a rate comparable to that observed in wild-type cells (see Figure 4 for representative examples from the ccs1 and ccs2 groups). The mature protein, however, comigrates with apocytochrome c₆ rather than with holocytochrome c₆. The newly synthesized apoprotein appears to be degraded rapidly, and very little conversion of apocytochrome c₆ to holocytochrome c₆ is evident for most strains (e.g., strain ccs2-5, Figure 4A). Occasionally, when a leaky ccs strain incorporates the radiolabel very well, it is possible to visualize the eventual, very slow conversion of a small amount of the apoprotein to the holoprotein, which accumulates (e.g., strain ccs2-4, Figure 4B, compare lanes 4 and 6). The pattern of labeling in this case is similar to that observed in gabaculine-treated wild-type cells, where maturation of pre-apocytochrome c₆ to apocytochrome c₆ is not affected, but conversion of apocytochrome c₆ to holocytochrome c₆ is slowed down because of depletion of heme, a substrate for the cytochrome c₆/heme lyase (Howe and Merchant 1994), and this pattern is consistent with our model, suggested in previous work, that heme attachment occurs after translocation on the luminal side of the thylakoid membrane. The severity of the acetate-requiring phenotype of each of the ccs mutants (which depends only on cyt f function) correlates reasonably, but not absolutely, with the ability of the strains to convert apocytochrome c₆ to holocytochrome c₆.

**Function of the nuclear CCS loci**: Formally, two types of functions may be proposed for the nuclear CCS loci: they may be required for expression of the ccsA gene, or they may function independently or in a complex with the ccsA gene product to catalyze a specific biochemical step required for heme attachment. To test whether ccsA was transcribed in the various ccs1-ccs4 mutant strains, RNA preparations from each strain were assayed for the presence of ccsA transcripts by a quantitative PCR-based method (Figure 5 shows representative examples Xie and Merchant 1996). Each nuclear mutant accumulates the ccsA transcript. The variation in the abundance of ccsA amplification products from different RNA preparations was not significant because the abundance of petA amplification products (used as an internal control) varied in parallel (data not shown). Also, the slight variations noted in ccsA transcript abundance were not reproducible between different members of the same complementation group. Thus, the CCS loci do not control the rate of synthesis or degradation of the ccsA transcript.

To test whether CcsA was synthesized in the mutant strains, pellet fractions from each mutant strain were tested by immunoblot analysis. Strains carrying ccs1 al-
leles do not accumulate CcsA, while strains carrying ccs2 alleles do (Figure 6). Although the amount of CcsA appears to be lower in ccs2 strains compared to the wild-type strain, the difference is probably not significant because other nonphotosynthetic strains (e.g., FUD7) also appear to accumulate less CcsA relative to the wild type. It is difficult to detect CcsA in extracts of ccs3-F18 and ccs4-F2D8 strains, but, occasionally, a signal can be detected in the form of a smeared band (Figure 6). This suggests that CcsA is synthesized in ccs3 and ccs4 strains, but it might be protease susceptible. In the case of ccs1 strains, at the present time, we cannot distinguish between the possibility that CcsA is not translated in ccs1 mutants and the possibility that CcsA is rendered protease susceptible in ccs1 mutants and therefore does not accumulate. The Ccs1 gene, which encodes a putative membrane-associated protein, was cloned recently (Inoue et al. 1997) and corresponds to the wild-type allele of the ccs1 locus described in this work (B. Dreyfuss and S. Merchant, unpublished results). On the basis of the distribution and arrangement of candidate CcsA and Ccs1 homologues in various organisms, and the probability that Ccs1 is a membrane protein, we favor the latter explanation (see discussion).

**DISCUSSION**

**Identification of mutants defective in chloroplast c-type cytochrome biogenesis:** In C. reinhardtii, a lesion in any one of the major subunits of the cytochrome b6/f complex prevents accumulation of the other subunits as well (Kuras and Wollman 1994). Thus, mutants that fail to accumulate the cytochrome b6/f complex could be defective at one of a myriad of different macromolecular processes involved in chloroplast mem-

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*Figure 3.*—Synthesis and degradation of cyt f in the ccs mutants. Synthesis of cyt f was assessed by immunoprecipitation of anti-cyt f-reactive polypeptides from solubilized acetone extracts of cells labeled for 10 min with Na235SO4 (lanes marked P). The labeled cells were sampled 40 min later after further incubation in the presence of unlabeled sulfate and chloramphenicol to assess the fate of the newly synthesized protein (lanes marked C). Immunoprecipitation of cyt f was essentially quantitative in the case of extracts from mutant strains (89-96% of the total). For the wild-type extracts, ~50% of the total cyt f was removed in the first immunoprecipitate. A second immunoprecipitate resulted in quantitative removal of cyt f. The signal shown corresponds to the first immunoprecipitate from extracts equivalent to 2 × 10^7 cells. For the samples shown in this figure, the specific activities (cpm/mg) of the “pulse” samples were the following: wild type (WT), 1.9; B6, 2.2; ccs1-4, 0.9; ccs1-1, 2.3; ccs3-F18, 4.2; ccs4-F2D8, 0.8. For the data shown in this figure, the dried fluorographs were exposed to Fuji Medical RX film as follows: WT, ccs1-4, ccs4-F2D8, 3 days; B6, ccs2, 7 days; ccs3-F18, 16 hr. For quantification of the amount of cyt f in each sample shown in this figure, the processing of each fluorograph was identical (with respect to exposure or developer time). The signal was estimated by densitometric scanning and was normalized for the specific activity of the sample.

*Figure 4.*—Synthesis and degradation of apocytochrome c6 in the ccs mutants. Synthesis of cyt c6 was assessed by immunoprecipitation of anti-cyt c6-reactive polypeptides from solubilized acetone extracts of cells labeled for 10 min (lanes marked P). The labeled cells were sampled at the indicated times (C10–C120) after further incubation (10–120 min) in the presence of unlabeled sulfate and cycloheximide to assess the fate of the newly synthesized proteins. The immunoprecipitate from extracts of wild-type cells (labeled for 10 min and further incubated for 10 min) is used as a holocytochrome c6 standard, while the immunoprecipitate from extracts of B6 cells (labeled for 10 min) is used as a standard for apocytochrome c6 and its precursors (Howe and Merchant 1994). The electrophoresis system described by Giulian et al. (1983) was used to resolve apo from holocytochrome c6. The dried, fluoro-infiltrated gels were exposed to Kodak XAR5 film for ~10 days. p, preapocytochrome c6; i, intermediate apocytochrome c6; a, apocytochrome c6; h, holocytochrome c6 (as in Howe and Merchant 1994). (A) Representative mutant strains from the ccs1 and ccs2 groups; (B) the high specific activity of the sample permits the visualization of a small amount of holocytochrome formed in this strain. The different intensity of labeling of cyt c6 for each strain (e.g., panel A vs. B) reflects the differences in the incorporation of precursor radiolabel into each strain.
brane biogenesis, including gene expression, protein translocation, cofactor biosynthesis, or complex assembly. In this work, we note that ~18% (or 188 of 1035 screened) of the metronidazole-enriched, acetate-requiring strains are affected in the accumulation of the cytochrome b_{6}/f complex. Because metronidazole treatment results in enrichment for electron transfer defects, the observed proportion is not surprising. For comparison, ~10% of the petite strains of S. cerevisiae are specifically defective in the cytochrome bc_{1} complex (Tzagoloff 1995).

In previous work, we demonstrated that a subset of the cytochrome b_{6}/f-deficient mutant strains were affected at the step of c-cytochrome assembly, and we further demonstrated that such mutants were also defective in cyt c_{6} accumulation. In this work, the dual cyt f / cyt c_{6} phenotype was exploited to identify rapidly 12 candidate heme attachment mutants among the collection of 201 cytochrome b_{6}/f-deficient strains. In each case, further biochemical analyses confirmed the assignment (see results), and we suggest, therefore, that pleiotropic cytochrome f and cyt c_{6} deficiencies are a hallmark of c-type cytochrome assembly mutants in C. reinhardtii. Genetic analysis of the mutants described in this article reveals a minimum of five loci involved in this process, ccsA and CCS1–CCS4. Although the ccsA, CCS1, and CCS2 groups have numerous representative alleles, the CCS3 and CCS4 groups have only one member each, which suggests that we have not yet saturated the CCS loci. Indeed, preliminary analysis of a new ccs strain generated recently by insertional mutagenesis indicates that it might define a sixth CCS locus (B. Dreyfuss and S. Merchant, unpublished results). While the proportion of ccs strains among the collection of cytochrome b_{6}/f-deficient mutants might not be surprising, the number of loci involved in this process certainly is.

**Mutations in ccsA:** The mutations in strains B6, ct34, and ct59 appear to be common in organelle genomes. Examples of similar mutations may be found in the FUD17 and acu-a-1-15 strains, where the function of the chloroplast atpE gene product is disrupted because of deletion of a single T in a sequence of six Ts (Robertson et al. 1990), in the FUD26 strain, where a 4-bp (TTAA) deletion from a duplicated AATTAATTAA sequence in the chloroplast psaB gene results in a non-photosynthetic phenotype (Girard-Bascou et al. 1987), and in the dum19 strains, where a respiration defect results from the deletion of a single T from three Ts in the mitochondrial COX1 gene (Colin et al. 1995). The fact that strains B6 and ct34 carry exactly the same mutation although they represent independent isolates from distinct mutagenesis experiments in different laboratories also speaks to the frequency of this type of mutation. In fact, phenotypic revertants can be readily isolated from the B6 strain, and they generally represent true revertants to the wild-type sequence (Xie and Merchant 1996).

**Genes required for cytochrome biogenesis in other systems:** Extensive genetic analyses of cytochrome-deficient mutants of S. cerevisiae revealed only two loci, CYC3 and CYT2, that are required for mitochondrial heme attachment during cytochrome c and c_{1} biosynthesis (Figure 7A). The gene products share ~30% sequence identity, but although they are proposed to catalyze similar reactions, one cannot substitute for the other. Thus, mutations at the CYC3 locus affect only cytochrome c and isocytochrome c assembly, while mutations at CYT2 affect only cytochrome c_{1} assembly (Matner and Sherman 1982; Dumont et al. 1987; Drygas et al. 1989; Stuart et al. 1990; Zoller et al. 1992). Candidate cytochrome c and c_{1} heme lyase homologues have been found in the Caenorhabditis elegans, mouse, and human genomes (Wilson et al. 1994; Schaefer et...
al. 1996), which suggests that mitochondrial c-type cytochrome biogenesis in these organisms resembles the pathway studied in *S. cerevisiae*.

In contrast to the system described above, genetic analysis of c-type cytochrome biogenesis in various bacteria led to the identification of a large number of genes whose products are required for conversion of apocytochromes c to their respective holoforms (Figure 7B). With the exception of HelX/CycY/CcmG, which contains a sequence motif (C—C) found in thioredoxins and protein-disulfide isomerases and exhibits thiol-dependent redox activity in vitro, the biochemical functions of the ccl/ hel/ cyc/ ccm gene products are not known, but analyses of sequence motifs and subcellular localization have led to reasonable predictions (Thony-Meyer et al. 1994; Loferer and Hennecke 1994; Kranz and Beckman 1995; Lang et al. 1996; Fabianek et al. 1997; Goldman et al. 1997; Monika et al. 1997). A supercomplex of multiple membrane and periplasmic proteins is envisioned to function as a c-type cytochrome assembly apparatus. This complex would catalyze the cytochrome c heme lyase reaction, maintain the substrates in a reduced form suitable for the ligation reaction, and also deliver heme from the cytoplasmic side and the apoprotein from the secretion apparatus to the subunit that catalyzes heme attachment in the bacterial periplasm. Homologues of some of the ccl, hel, and cyc genes are found in plant mitochondrial genomes (Oda et al. 1992; Gonzalez et al. 1993; Schuster et al. 1993; Schuster 1994; Jekabsons and Schuster 1995; Handa et al. 1996; Unsell et al. 1997), and a cDNA encoding a candidate CycJ homologue with a putative mitochondrial targeting signal peptide was cloned from *A. thaliana* (EMBL/GenBank accession number U72502), which suggests that c-type cytochrome assembly in plant mitochondria may use a machinery that is similar to the one in most proteobacteria. Because Ccl/Hel/Cyc/Ccm homologues are not found in the *S. cerevisiae* genome, and candidate homologues in cyanobacteria, chromatophyte algae, and other gram-positive bacteria. Each gene is indicated by a rectangle. Candidate or known homologues are presented by the same fill pattern. A horizontal line indicates that multiple genes are organized at a single locus. The gene names are indicated above the rectangles, and for eukaryotic species, genes encoded in each genome are indicated as follows: nu, nuclear; mt, mitochondria; cp, chloroplast. Rectangles without names are classified only as open reading frames in the databases. In chloroplast-containing organisms, genes for two pathways (B and C) must be found—one for the chloroplast cytochromes and one for the mitochondrial cytochromes.

![Figure 7](image-url) —Three types of c-type cytochrome biogenesis systems. (A) Cytochrome biogenesis genes required for holocytochrome c and c₃ formation in the mitochondria of fungi, mammals, and a nematode (*C. elegans*). One gene is required for holocytochrome c formation, and a different gene is required for holocytochrome c₃ formation in *S. cerevisiae* (CYC3 and CYT2, respectively). (B) Related operons containing multiple genes, each of which is required for c-type cytochrome biogenesis in several proteobacteria, and candidate organelle- and nucleus-encoded homologues that might function in plant mitochondrial c-type cytochrome biogenesis. (C) The arrangement and relationship of genes required for c-type cytochrome biogenesis in chloroplasts (Ccs1 and ccsA), and a gram-positive bacterium (ccdA) and candidate homologues in cyanobacteria, chromatophyte algae, and other gram-positive bacteria. Each gene is indicated by a rectangle. Candidate or known homologues are presented by the same fill pattern. A horizontal line indicates that multiple genes are organized at a single locus. The gene names are indicated above the rectangles, and for eukaryotic species, genes encoded in each genome are indicated as follows: nu, nuclear; mt, mitochondria; cp, chloroplast. Rectangles without names are classified only as open reading frames in the databases. In chloroplast-containing organisms, genes for two pathways (B and C) must be found—one for the chloroplast cytochromes and one for the mitochondrial cytochromes.

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C. reinhardtii

P. purpurea

Synechocystis sp. 6803

M. leprae/tuberculosis

B. subtilis

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The CCS genes correspond to a third cytochrome biogenesis pathway. The involvement of multiple CCS loci in chloroplast cytochrome c biogenesis suggests that the chloroplast pathway might be more similar to the bacterial pathway rather than the yeast mitochondrial pathway. Furthermore, heme attachment in chloroplasts occurs in the lumen, which is topologically analogous to the bacterial periplasm. Therefore, one might predict that the C. reinhardtii CCS loci would encode homologues of the cyc/ ccl/ hel gene products of rhizobia and Rhodobacter spp. We would also expect that candidate cyanobacterial homologues of the cyc/ ccl/ hel gene products might be identified in the genome database, and those would correspond to the C. reinhardtii Ccs genes. Nevertheless, candidate homologues of the cyc/ ccl/ hel gene products are not encoded in the genome of Synechocys-
tis sp. 6803 (Kaneko et al. 1996). Rather, the Synecocys-
tis sp. 6803 genome encodes homologues of CcsA and
Ccsl, as well as a novel cytochrome biogenesis protein,
CcdA, of Bacillus subtilis (Figure 7C). Analyses of the
distribution of ccsA-, Ccs1, and ccdA-like sequences (Xie
and Merchant 1996; Schiott et al. 1997; Inoue et al.
1997) in the genome databases and the relationships
between candidate homologues suggest that the Ccs
genes are likely to be distinct from the ccc/ ccl/ hcl/ ccm
genes found in most proteobacteria.

Therefore, we propose that the Ccs genes define a
third pathway for c-type cytochrome biogenesis, which
is found in chloroplasts, cyanobacteria, and several
gram-positive bacteria (Figure 7C). The third pathway
may be completely distinct from the pathway requiring
ccl/ hcl/ ccc/ ccm gene products, or it may have the same
origin as the ccl/ hcl/ ccc/ ccm pathway, but the relation-
ship between some components might be unidentifiable
at the level of sequence comparison because of rapid
divergence. The fact that c-type cytochromes from B.
subtilis and Synecocystis sp. 6803 can be as-
sembled in the Escherichia coli periplasm is not inconsis-
tent with the latter hypothesis (von Wachenfeldt and
Hederstedt 1990; Diaz et al. 1994). The cloning of the
CCS2–CCS4 loci will certainly lead to a better under-
standing of c-type cytochrome assembly in chloroplasts
and gram-positive bacteria.

Function of Ccsl: Two models can be proposed to
explain why CcsA does not accumulate in ccs1 strains
(Figure 6). CcsA might not be translated if Ccs1 is a
nuclear regulator of ccsA translation, or, if Ccs1 interacts
with CcsA in a multisubunit cytochrome complex, the
absence of Ccs1 might result in destabilization of the
complex and loss of CcsA because of degrada-
tion. Because Ccs1 is conserved but plastid-encoded in
two other algae, it seems unlikely that it would function
as a regulator. Also, the fact that a Ccs1 homologue is
found in most proteobacteria (Figure 6C). The third pathway
is found in chloroplasts, cyanobacteria, and several
gram-positive bacteria (Figure 7C). The third pathway
may be completely distinct from the pathway requiring
ccl/ hcl/ ccc/ ccm gene products, or it may have the same
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tion. Because Ccs1 is conserved but plastid-encoded in
two other algae, it seems unlikely that it would function
as a regulator. Also, the fact that a Ccs1 homologue is
found in several respiring bacteria where the gene regu-
latory mechanisms might be expected to be quite dis-
tinct from those operating in a eukaryotic photosyn-
thetic thylakoid argues against a regulatory function
for Ccsl. Finally, the operon-like arrangement of Ccsl-
and ccsA-like genes in the gram-positive bacteria is con-
sistent with the model that Ccsl and CcsA function in
the same pathway, perhaps as subunits of a multicom-
ponent complex. In Helicobacter pylori, candidate Ccsl-
and CcsA-homologues are encoded in a single open
reading frame (HP0378: EMBL/Genbank accession
number AE000511).

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LITERATURE CITED

Beckman, D. L., and R. G. Kranz, 1993 Cytochromes c biogenesis
in a photosynthetic bacterium requires a periplasmic thio-

Beckman, D. L., D. R. Trawick and R. G. Kranz, 1992 Bacterial cy-

Bennoun, P., and P. Delepelaire, 1982 Isolation of photosynthesis
mutants in Chlamydomonas, pp. 25–38 in M ethods in Chloroplast Mo-

cular Biology, edited by M. Edelman, R. B. Hallick and N.-H.

parental mutants of Chlamydomonas reinhardtii defective in photo-
synthesis, pp. 721–726 in Chloroplast Development, edited by A.
Kooyongulou and J. H. Argyroudi-Akoyongulou. Elsevier/
North-Holland Biomedical Press, Amsterdam.

Bennoun, P., A. Masson and M. Delosme, 1980 A method for com-
plementary analysis of nuclear and chloroplast mutants of

Chen, X. K., L. Kindle and D. B. Stern, 1995 The initiation codon
determines the efficiency but not the site of translation initiation

Colin, M., M.-P. Dorthu, F. Duby, C. Remacle, M. Dinant et al.,
1995 Mutations affecting the mitochondrial genes encoding
the cytochrome oxidase subunit I and apocytochrome b of

Diaz, A. F., Navarro, M. Hervas, J. A. Navarro, S. Chavez et al.,
1994 Cloning and the correct expression in E. coli of the pet
gene encoding cytochrome c, from Synecocystis 6803. FEBS Lett.

Drygas, M. E., A. M. Lambowitz and F. E. Nargang, 1989 Cloning
and analysis of the Neurospora crassa gene for cytochrome c

and F. Sherman, 1987 Identification and sequence of the gene encoding cyto-

chore c heme lyase in the yeast Saccharomyces cerevisiae. EMBO J.

Fabianek, R. A., M. Huber-Wunderlich, R. Glöckshuber, P. Kunzler,
H. Hennecke et al., 1997 Characterization of the Bradyrhiz-
obiunum japonicum Cyt-c oxidase, a membrane-anchored periplasmic
thioredoxin that may play a role as a reductant in the biogenesis
development for analysis and quantitation of proteins on one-dimen-

Goldman, B. S., D. L. Beckman, A. Bali, E. M. Monika, K. K.
Gabbert et al., 1997 Molecular and immunological analysis of an
ABC transporter complex required for cytochrome c biogene-


Goldschmidt-Clermont, M. J., Girard-Bascou, Y. Choquet and J.
D. Rochaix, 1990 Transsplicing mutants of Chlamydomonas

Gonzalez, D. H., G. Bonnard and J.-M. Grienberger, 1993 A

gene involved in the biogenesis of cytochromes is co-transcribed

Gorman, D. S., and R. P. Levine, 1966 The photosynthetic electron
transport chain of Chlamydomonas reinhardtii. VI. Electron trans-
port in mutant strains lacking either cytochrome 553 or plastocya-

Handa, H., G. Bonnard and J.-M. Grienberger, 1996 The rape-
seed mitochondrial gene encoding the protein homologue to
Ccsl is divided in two independently transcribed reading frames.


1993 Expression, purification and characterization of a putative human homolog of cytochrome c-typ synthetase gene (HCCS) isolated from the critical region for microphthalmia with linear skin defect (MLS). Genomics 34: 166–172.


