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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Manuscript received June 6, 1997
Accepted for publication October 17, 1997

ABSTRACT

Chloroplasts contain up to two *c*-type cytochromes, membrane-anchored cytochrome *f* and soluble cytochrome c_6. 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 nucleus-encoded cytochrome c_6 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 c_6. 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 CxxCH sequence near the amino terminus of the protein. Cytochrome c_1 and *c* in mitochondria and respiring bacteria function to oxidize quinols and reduce a terminal oxidase, while analogous cytochromes in chloroplasts (cytochromes *f* and c_6) 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 c_1 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 c_1, respectively, and it was suggested that these loci encoded cytochrome *c* and c_1/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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tachment 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* (*hel*, *ccl* loci), *Bradyrhizobium japonicum* (*cyc* loci), and *Paracoccus denitrificans* by complementation of the mutant phenotypes (Kranz 1989; Ramseier *et al.* 1991; Beckman *et al.* 1992; Beckman and Kranz 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 (Willey 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 *petA* 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 *ccs3-F18*), and F2D8 (now *ccs4-F2D8*) 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*, *ccs2-1*, *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° in TAP medium (Harris 1989) under cool fluorescent lights (15–125 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with agitation (225 rpm). Mutant strains were grown under the same conditions, except that the illumination was always reduced (15–25 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

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 fluorodeoxyuridine and enrichment with metronidazole, as described by Bennoun *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 ($\sim$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 ε-aminocaproic acid, and 1 mm benzamidine, and were either analyzed immediately or stored frozen for future analysis. Before analysis, pellet fractions equivalent to 6 μg of chlorophyll were collected by centrifugation, resuspended in lysis buffer [62.5 mm Tris-Cl (pH 6.8), 2% sodium dodecyl sulfate, 20% glycerol, 5% 2-mercaptoethanol plus protease inhibitors as described above], and heated to 90° 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 retested 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 PSII, and cyt c_6. For

the initial screen for the cytochrome c_6 deficiency, candidate strains were grown on copper-deficient TAP agar (1.5%) slabs (Quinn and Merchant 1995), and soluble extracts were tested for cyt c_6 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 c_6. All strains were tested routinely for their fluorescence properties (see above) to confirm that they displayed a characteristic cytochrome b_6/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 tetrads). 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 H_2O to eliminate unmated gametes. Fluorescence measurements to reveal the presence or absence of cytochrome b_6/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 immunodecoration 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; Chen *et al.* 1995; Xie and Merchant 1996) were diluted as follows: antiplastocyanin (1:5000), anti-cyt c_6 (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 Tris-buffered saline, incubated with the reagents for the chemiluminescence assay (Supersignal; 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 *Nde*I–*Hin*dIII 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 *ccs1–ccs4* strains, the procedure was modified to ensure that the conditions were suitable for quantitative estimation of transcript abundance. Specifically, a random primer pdN_6 (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-min extension at 72°. The abundance of the *petA* transcript was estimated simultaneously by amplification with primers cytf-1 (5′-TTACCAGCTGCTGAT GCG-3′) and cytf-5 (5′-AACGAACTGGAATCCCCTTATAG-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 pdN_6 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 c_6 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 c_6, 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 IgGSORB in immunoprecipitation buffer [40 mm Tris-Cl (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 IgGSORB plus nonspecifically bound proteins were removed by centrifugation (12,000 *g*, 5 min). Aprotinin (10 μl of 5 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% IgGSORB solution. After removal of the antigen-antibody complex with IgGSORB, the supernatant was retreated with anti-cyt *f* to assess the effectiveness of the immunoprecipitation. The IgGSORB-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 IgGSORB into 0.1 ml of solution A by heating at 95° for 5 min (Li *et al.* 1996). The IgGSORB 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 ^{35}S into acid-insoluble material.

Cyt c_6 was immunoprecipitated as described previously (Howe and Merchant 1992; Li *et al.* 1996), except that the samples were not subject to any preabsorption. Antiserum (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 c_6 was immunoprecipitated from labeled wild-type cells (10 min of radiolabeling followed by 10 min of incubation with unlabeled sulfate) to generate a holocytochrome c_6 standard sample, and an apocytochrome c_6 standard was generated by immunoprecipitation of anti-cyt c_6-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 c_6^- 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 b_6/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 b_6/f complex are blocked in the electron flow beyond the plastoquinol pool, and this results in a characteristic fluorescence signature (Bennoun and Delepelaire 1982). Candidate cytochrome b_6/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 b_6/f complex were chosen for further study. For the acetate-requiring strains resulting from UV mutagenesis and metronidazole enrichment, cytochrome b_6/f deficiencies were identified by the estimation of anti-cyt *f*-reactive polypeptides 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 b_6/f-deficient strains totalled 201.

In previous work, we showed that among the cytochrome b_6/f-deficient mutant strains, a subgroup that lacked cyt c_6 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 c_6 accumulation. Twelve of the 201 cytochrome b_6/f-deficient strains displayed severe defects in cyt c_6 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 c_6 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 c_6 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 *ccs 1-2*, *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 c_6 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 ($t_{1/2} \sim$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 b_6/f complex and cyt c_6. 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*,

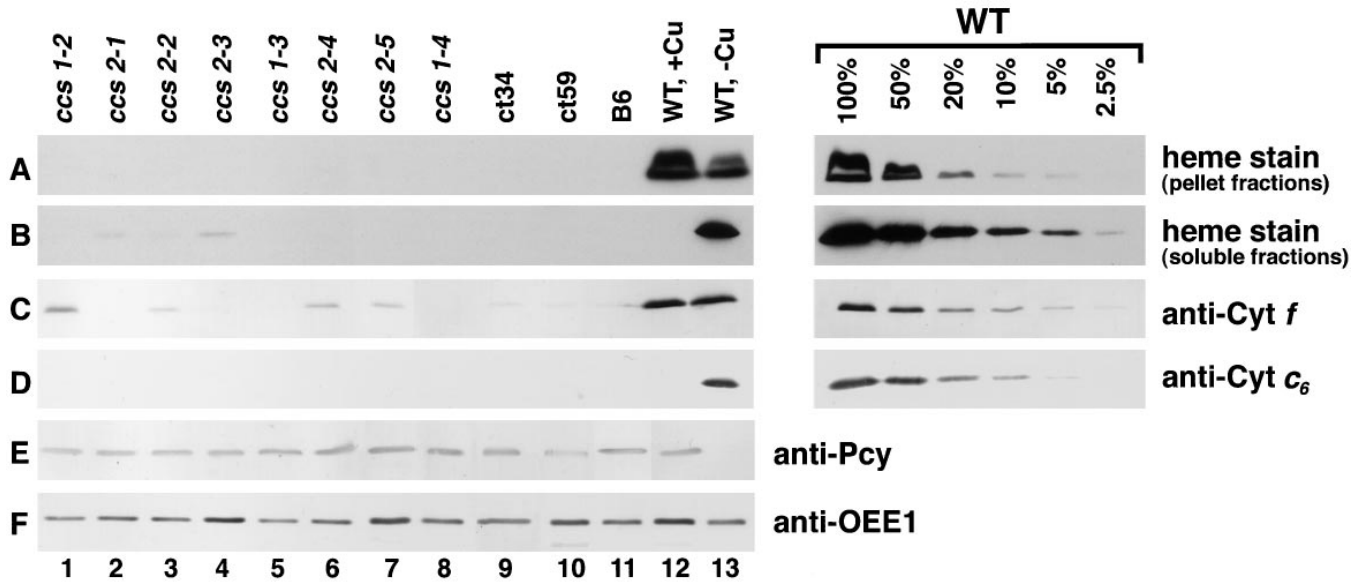

Figure 1.—Accumulation of *c*-type cytochromes and other lumenal proteins. Protein fractions were prepared from copper-supplemented [for detection of plastocyanin (E), cyt *f* (A and C), and OEE1 (F)] or copper-deficient [for cyt c_6 (B and D)] cultures of each strain. The supernatant fraction was analyzed for cyt c_6, plastocyanin, and OEE1, while the pellet fraction was analyzed for cyt *f* abundance. Samples corresponding to 5 μg chlorophyll were transferred to nitrocellulose membranes after electrophoresis through SDS-containing acrylamide (12%) gels and were analyzed by heme stain (2-min treatment with the reagent) or immunodecoration. The exposure shown for the heme stain is the second exposure (1.5 min for soluble or 35 min for pellet fractions), and it was set up 10 min after treatment of the membrane with the reagent. When the signal is captured immediately after exposure to the reagent, <1% of wild-type levels of holocytochrome c_6 and holocytochrome *f* can be detected. The dilution series for heme stain and immunodecoration was developed in parallel with the experimental samples from the mutant strains. The results shown here are from a single representative experiment where each analysis was performed on the same extract.

cyt b_6, 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

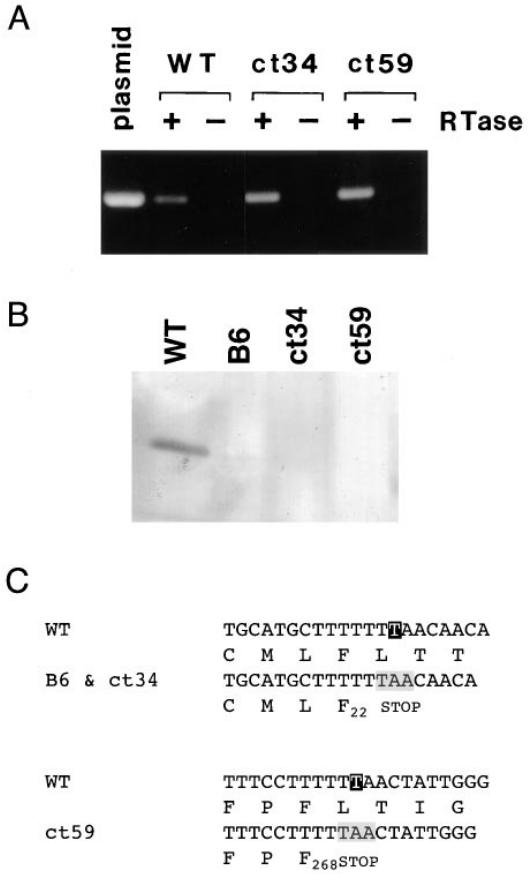

Figure 2.—Expression of *ccsA* in strains ct34 and ct59. (A) Total RNA was isolated from wild-type and mutant strains, digested with RQ1 DNase (Promega, Madison, WI), and used as a template for reverse transcription (5 μg per reaction). The cDNA was detected by amplification with *ccsA*-specific primers (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 polyvinyldifluoride 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 subscripted 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 c_6 in the *ccs* mutants: To confirm that the mutants were able to synthesize cyt *f* and cyt c_6, 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 *cycH* locus in *Rhodobacter capsulatus* showed that certain *cycH* alleles displayed differential effects on the maturation of membrane *vs.* soluble cytochromes (Lang *et al.* 1996). Thus, each mutant strain

TABLE 1

Complementation analysis of cyt f^-/cyt c_6^- strains reveals four nuclear loci

		ac206	*1-2*	*1-3*	*1-4*	*2-1*	*2-2*	*2-3*	*2-4*	*2-5*	*F18*	*F2D8*
	ac206	0/22	−	−	−	+	+	+	+	+	+	+
CCS1	*1-2*	0/19	0/12	−	−	+	+	+	+	+	+	+
	1-3	0/38	0/23	0/24	−	+	+	+	+	+	+	+
	1-4	0/13	0/20	0/36	0/26	+	+	+	+	+	+	+
	2-1	21/25	19/29	22/30	13/21	0/12	−	−	−	−	+	+
	2-2	11/27	17/25	17/22	15/24	0/27	0/17	−	−	−	+	+
CCS2	*2-3*	20/25	6/16	8/29	23/28	0/39	0/30	0/21	−	−	+	+
	2-4	20/22	18/28	18/38	6/20	0/17	0/24	0/17	0/22	−	+	+
	2-5	4/20	18/25	9/24	18/20	5/27[a]	6/35[a]	0/37	0/29	3/23[a]	+	+
						0/21	5/30[a]			0/29		
CCS3	*F18*	18/22	11/15	18/23	17/23	30/54	11/24	20/25	12/26	14/23	0/27	+
CCS4	*F2D8*	9/21	13/20	8/18	25/29	11/22	14/26	24/28	7/27	10/27	9/19	0/16

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 $a1/(a2 \times b1/b2)$, where *a1* is the number of zygotes that germinated and gave rise to colonies on minimal medium, *a2* is the number of zygotes transferred to minimal medium, *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.

[a] 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 c_6 synthesis. Pulse-radiolabeling studies indicated that cyt c_6 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 c_6 rather than with holocytochrome c_6. The newly synthesized apoprotein appears to be degraded rapidly, and very little conversion of apocytochrome c_6 to holocytochrome c_6 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 holoform, 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 c_6 to apocytochrome c_6 is not affected, but conversion of apocytochrome c_6 to holocytochrome c_6 is slowed down because of depletion of heme, a substrate for the cytochrome *c*/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 lumen 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 c_6 to holocytochrome c_6.

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-

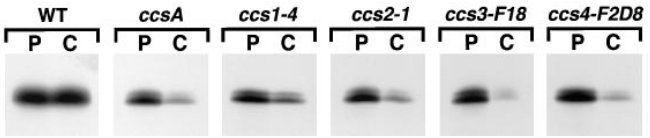

Figure 3.—Synthesis and degradation of cyt *f* in the *ccs* mutants. Synthesis of cyt *f* was assessed by immunoprecipitation of anti-cyt *f*-reactive polypeptides from solubilized acetone extracts of cells labeled for 10 min with $Na_2{}^{35}SO_4$ (lanes marked P). The labeled cells were sampled 40 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/cell) of the "pulse" samples were the following: wild type (WT), 1.9; B6, 2.2; *ccs1-4*, 0.9; *ccs2-1*, 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, *ccs2-1*, 2 days; *ccs3-F18*, 16 hr. For quantitation 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.

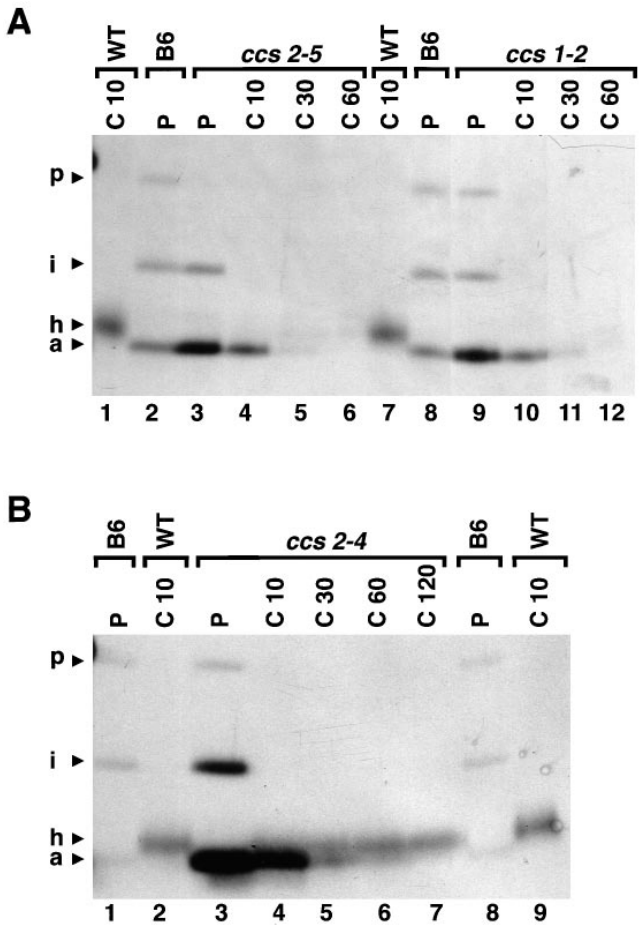

Figure 4.—Synthesis and degradation of apocytochrome c_6 in the *ccs* mutants. Synthesis of cyt c_6 was assessed by immunoprecipitation of anti-cyt c_6-reactive polypeptides from solubilized acetone extracts of cells labeled for 10 min (lanes marked P). The labeled cells were sampled at the indicated 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 c_6 standard, while the immunoprecipitate from extracts of B6 cells (labeled for 10 min) is used as a standard for apocytochrome c_6 and its precursors (Howe and Merchant 1994). The electrophoresis system described by Giulian *et al.* (1983) was used to resolve apo from holocytochrome c_6. The dried, fluor-infiltrated gels were exposed to Kodak XAR5 film for ~10 days. p, preapocytchrome c_6; i, intermediate apocytochrome c_6; a, apocytochrome c_6; h, holocytochrome c_6 (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 c_6 for each strain (*e.g.*, panel A *vs.* B) reflects the differences in the incorporation of precursor radiolabel into each strain.

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 b_6/f complex prevents accumulation of the other subunits as well (Kuras and Wollman 1994). Thus, mutants that fail to accumulate the cytochrome b_6/f complex could be defective at one of a myriad of different macromolecular processes involved in chloroplast mem-

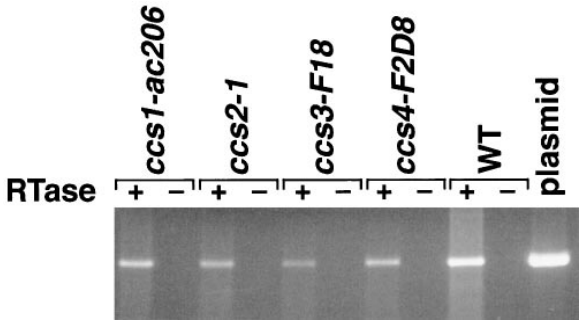

Figure 5.—Accumulation of *ccsA* transcripts in the nuclear *ccs* mutants. Total RNA was isolated from wild-type and mutant strains, digested with RQ1 DNase (Promega), and used as a template for reverse transcription (1 μg per reaction). The cDNA corresponding to *ccsA* transcripts was detected by amplification 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). A cDNA corresponding to *petA* was amplified in parallel as an internal control (not shown). Details of the assay are provided in materials and methods.

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 cyt *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 *CC4* 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.

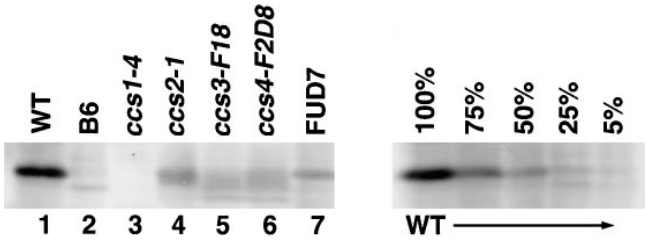

Figure 6.—Reduced abundance of CcsA in the nuclear *ccs* mutants. Pellet fractions (corresponding to 20 μg chlorophyll) of protein preparations from wild-type or mutant *ccs* strains (as indicated) were tested for the presence of CcsA by immunoblot analysis (as described in legend to Figure 2).

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 *ac-u-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 nonphotosynthetic 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; Zollner *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*

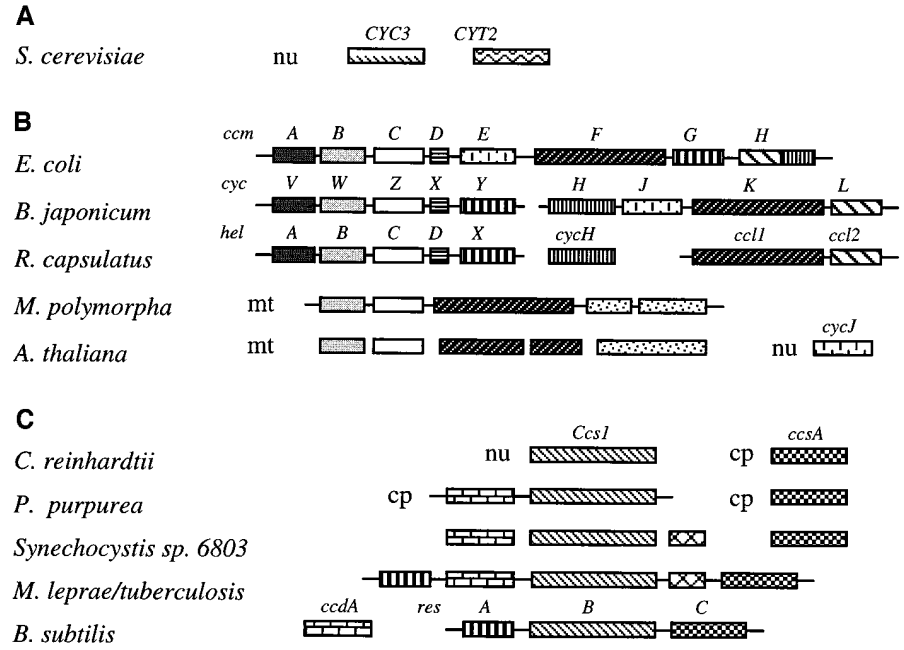

Figure 7.—Three types of *c*-type cytochrome biogenesis systems. (A) Cytochrome biogenesis genes required for holocytochrome *c* and c_1 formation in the mitochondria of fungi, mammals, and a nematode (*C. elegans*). One gene is required for holocytochrome *c* formation, and a different gene is required for holocytochrome c_1 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, chromophyte 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.

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; Unseld *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 of the yeast *CYC3* and *CYT2* genes are not found in the bacterial genomes, it seems that the two pathways are distinct.

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* genes 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 Synechocystis sp. 6803 genome encodes homologues of CcsA and Ccs1, 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 *cyc*/*ccl*/*hel*/*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*/*hel*/*cyc*/*ccm* gene products, or it may have the same origin as the *ccl*/*hel*/*cyc*/*ccm* pathway, but the relationship 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 Synechocystis sp. 6803 can be assembled in the *Escherichia coli* periplasm is not inconsistent 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 understanding of *c*-type cytochrome assembly in chloroplasts and gram-positive bacteria.

Function of *Ccs1*: 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 assembly complex, the absence of Ccs1 might result in destabilization of the complex and loss of CcsA because of degradation. 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 regulatory mechanisms might be expected to be quite distinct from those operating in a eukaryotic photosynthetic compartment argues against a regulatory function for Ccs1. Finally, the operon-like arrangement of *Ccs1*- and *ccsA*-like genes in the gram-positive bacteria is consistent with the model that Ccs1 and CcsA function in the same pathway, perhaps as subunits of a multicomponent complex. In *Helicobacter pylori*, candidate CcsA- and Ccs1-homologues are encoded in a single open reading frame (HP0378: EMBL/Genbank accession number AE000511).

We thank Prof. Charles Marshall, Drs. Hong Hua Li and Jeanette Quinn, Gloria Turner and Jeffery Moseley (UCLA), and the members of the UPR9072 laboratory in Paris for helpful discussions. The research at UCLA was supported by the National Institutes of Health (NIH) (GM-48350). Z.X. was supported by fellowships from the Graduate Division at UCLA (Ursula Mandel Fellowship and Newman Dissertation Year Fellowship) and an NIH Predoctoral Award in Biotechnology (GM-08375), B.W.D. is supported by an NIH-NRSA (GM-17483), and S.M. is supported by an NIH Research Career Development Award (GM-00594). Work at Institut de Biologie Physico-Chimique was supported by the Centre National de la Recherche Scientifique, Unité Propre de Recherche 9072 du Centre National de Recherche Scientifique (France). R.K. was supported by a grant from the Ministere de l'Education et de la Recherche.

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Communicating editor: K. J. Newton