Ccs1, a Nuclear Gene Required for the Post-translational Assembly of Chloroplast c-Type Cytochromes*

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Nuclear genes play important regulatory roles in the biogenesis of the photosynthetic apparatus of eukaryotic cells by encoding factors that control steps ranging from chloroplast gene transcription to post-translational processes. However, the identities of these genes and the mechanisms by which they govern these processes are largely unknown. By using glass bead-mediated transformation to generate insertion mutations in the nuclear genome of Chlamydomonas reinhardtii, we have generated four mutants that are defective in the accumulation of the cytochrome b6f complex. One of them, strain abf3, also fails to accumulate holocytchrome c6. We have isolated a gene, Ccs1, from a C. reinhardtii genomic library that complements both the cytochrome b6f and cytochrome c6 deficiencies in abf3. The predicted protein product displays significant identity with Ycf44 from the brown alga Odontella sinensis, the red alga Porphyra purpurea, and the cyanobacterium Synechocystis strain PCC 6803 (25–33% identity). In addition, we note limited sequence similarity with ResB of Bacillus subtilis and an open reading frame in a homologous operon in Mycobacterium leprae (11–12% identity). On the basis of the pleiotropic c-type cytochrome deficiency in the ccs1 mutant, the predicted plastid localization of the protein, and its relationship to candidate cytochrome biosynthesis proteins in Gram-positive bacteria, we conclude that Ccs1 encodes a protein that is required for chloroplast c-type holocytchrome formation.

Studies in the unicellular green alga Chlamydomonas reinhardtii and vascular plants have demonstrated that the proteins required for photosynthesis are encoded by genes that reside in two distinct cellular compartments: the nucleus and the chloroplast (reviewed in Refs. 1–3). The nucleus encodes both structural polypeptides and proteins that play pivotal roles in the regulation of chloroplast gene expression (4–10) and in the maturation and assembly of the photosynthetic apparatus (11, 12). Although biochemical and genetic studies are beginning to lead to a better understanding of the roles that nucleus-encoded regulatory proteins play in these processes (7, 8, 10, 13), the identities of many of these proteins and the mechanisms by which they exert their influence remain largely unknown.

Defects in genes that regulate chloroplast gene expression or that are involved in the maturation or assembly of photosynthetic complexes are likely to cause a nonphotosynthetic phenotype. C. reinhardtii is an attractive organism for studying these genes, because nonphotosynthetic mutants are viable when provided with acetate as a carbon and energy source. With the ultimate goal of understanding the mechanisms by which nucleus-encoded factors regulate chloroplast gene expression and function, we have generated tagged nonphotosynthetic mutants, focusing initially on the isolation and characterization of nuclear genes required for the biogenesis of the cytochrome b6f complex of C. reinhardtii.

The cytochrome b6f complex carries out photosynthetic electron transfer between photosystem (PS) II and PS I and is composed of both chloroplast- and nucleus-encoded subunits (14, 15). Its compositional simplicity relative to the PS II and PS I complexes makes it an attractive model for studying the assembly of chloroplast energy-transducing complexes. The chloroplast-encoded subunits of the cytochrome b6f complex include products of petA (cytochrome f), petB (cytochrome b6), petD (subunit IV), and two 4-kDa proteins encoded by the petG and petL genes (15–20). The nucleus-encoded components include the Rieske Fe-S protein (product of PetC; Ref. 21) and a 4-kDa protein (product of PetM; Refs. 22 and 23). The binding sites for the hemes and the Fe–S2 center are well defined, and each cofactor binding site lies within a single polypeptide. This feature makes the b6f complex particularly suited for studies of cofactor-polypeptide association and assembly.

Previous studies have identified a number of nuclear loci required for the biogenesis of the cytochrome b6f complex (9, 11, 14, 24, 25). Among these are a distinct subset of four nuclear loci that are also required for synthesis of cytochrome c6. Cytochrome c6, a functional substitute for plastocyanin, is a soluble heme-containing protein that carries out electron transfer between the cytochrome b6f complex and PS f; its expression is induced under copper-deficient conditions (11, 26). Cytochrome c6 and cytochrome f are c-type cytochromes in

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1 The abbreviations used are: PS, photosystem; bp, base pair(s); kb, kilobase pair(s); ORF, open reading frame; RFLP, restriction fragment length polymorphism; SGII, Sager-Granick medium; SuIV, subunit IV.

which the heme prosthetic group is covalently attached to the apoproteins. While cytochrome \( f \) is chloroplast-encoded, cytochrome \( c_6 \) is encoded by the nuclear Cyc6 gene, synthesized in the cytosol, and imported post-translationally into the chloroplast (26, 27). Thus, there are few biosynthetic steps that are common for cytochrome \( c_6 \) and cytochrome \( f \) besides translocation across the thylakoid membrane, luminal processing, and heme attachment.

In previous work, we identified several mutants that display a cytochrome \( b_{5f} \) /cytochrome \( c_6 \) phenotype, and biochemical characterization confirmed that the strains were able to translocate and process precursor and intermediate forms of cytochrome \( f \) and \( c_6 \) but were unable to convert the apocytochromes to their respective holohemoproteins (11, 28). This work defined a specific biochemical phenotype for heme attachment mutants (cytochrome \( b_{5f} \) /cytochrome \( c_6 \) phenotype) and suggested that a common set of proteins is required for holocytchrome formation in the thylakoid lumen. One of these is the product of the chloroplast-encoded \( ccsA \) gene, which is required for the biosynthesis and accumulation of the cytochrome \( b_{5f} \) complex and cytochrome \( c_6 \) (29). Loss of \( ccsA \) function results in a defect at the step of heme attachment to apocytochromes \( c_6 \) and \( f \).

In the present study, we describe the isolation and characterization of a nuclear gene, \( Ccs1 \) (\( c \)-type cytochrome synthesis), which is required for assembly of the cytochrome \( b_{5f} \) complex and cytochrome \( c_6 \). On the basis of a pleiotropic \( c \)-type cytochrome deficiency in a strain lacking functional \( Ccs1 \), we suggest that its gene product functions in the same pathway as \( CcsA \).

**EXPERIMENTAL PROCEDURES**

**Strains—** \( C. reinhardtii \) strain nit1-305 cu15, which is the wild type for photosynthesis, was used as a recipient for nuclear transformations. \( C. reinhardtii \) strain CC-125 (wild type) was obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). Strain B6, isolated by Gal et al. (30), does not accumulate cytochrome \( c_6 \) or the cytochrome \( b_{5f} \) complex due to a frameshift mutation in the chloroplast \( ccsA \) gene (11, 29, 31). CC-125 and B6 were used as positive and negative control strains, respectively, for the analyses of cytochrome \( c_6 \) and cytochrome \( f \) synthesis. \( E. coli \) strains XL1-Blue and DH5\( \alpha \) were used as hosts for recombinant DNA techniques (32).

**Transformation and Screening for Nonphotosynthetic Mutants—** Cells were grown in Sager-Granick medium (33) supplemented with ammonium nitrate (SGII-NH\(_4\)) under continuous light (50 μEin). Cells were grown in Sager-Granick medium (33) supplemented with potassium nitrate (SGII-NO\(_3\)) and grown under dim light (500 μEin).

**Preparation and Analysis of Nucleic Acids—** Isolation of plasmid DNA was performed either by the boiling method (40) or by a modified alkaline lysis procedure (41). \( C. reinhardtii \) DNA was isolated as described previously (27), while poly(A\(^+\)) RNA was isolated as described previously (44). Southern blotting and hybridization of \( C. reinhardtii \) DNA to radioactive probes was performed as described previously (35). RNA blot hybridization was performed as described by Hill et al. (45). petA mRNAs were identified by hybridization to a 600-bp HindIII/AscI radiolabeled fragment from plasmid pH4A.6 (16), and mRNAs encoding cytochrome \( c_6 \) (Cyc6) were identified by hybridization to a 710-bp EcoRI radiolabeled fragment from plasmid pGEM1Cre562 7A-4 (46). The Bsc82-specific probe consisted of the 0.8-kb EcoRI dDNA fragment of the plasmid p149A, which was obtained from the Chlamydomonas Genetics Center. 5'-GCGGTACTGGATCCGAGATGAGGAGG-3' and 5'-CCGGGGATCCGTGGTGCGGGG-3', the Superscript Preamplification System (Life Technologies, Inc.) according to the manufacturer's instructions.

**Plasmid Rescue and Screening of Genomic and cDNA Clones—** Genomic DNA was digested with Sau3A, ligated, and transformed by electroporation into \( E. coli \) (47). A fragment from the rescued plasmid was used as a probe to screen a \( C. reinhardtii \) genomic library comprising strain 21gr DNA in AFXII (Stratagene, La Jolla, CA); this library was obtained from Dr. R. Schnell and Dr. P. Lefebvre (University of Minnesota, St. Paul, MN; Ref. 48).

A \( C. reinhardtii \) cDNA library made from RNA isolated from vegetative cells of strain CC-621 in AAZP II (Stratagene) was obtained from Dr. J. Woessner and Dr. U. Goodenough (Washington University, St. Louis, MO; Ref. 49). The library (8 × 10\(^6\) independent plagues) was screened with a mixture of probes made from the 0.45-kb SphI and 1.5-kb SphII/RpI fragments (probes 2 and 3, Fig. 4) of pCes1–1. Since we were unable to convert the cDNA insert from the single positive plaque into a plasmid by superinfection with a helper, the cDNA insert was then isolated from a single plaque (3 cycles of the following: 92 °C for 30 s; 45 °C for 1 min; 72 °C for 2 min) using T3 and T7 primers. The fragment was digested with EcoRI/SalI and XhoI/ScalI to produce 0.5-kb ScalI/EcoRI and 2.0-kb XhoI/ScalI fragments, which were then subcloned into pBluescript KSII+ (the ScalI site is located within the cDNA, while the terminal EcoRI and XhoI sites are derived from the AZAP II vector).
Ccs1 Required for Assembly of Chloroplast c-type Cytochromes

FIG. 1. Accumulation of chloroplast-encoded subunits of the cytochrome b6f complex. Western blots of total cell extracts of the wild type (WT) and nonphotosynthetic mutants (abf3, -9, -30, and -34) were probed with antibodies against cytochrome (Cyt f), cytochrome b6, SuIV, and the D2 protein of PS II. The ΔpetA and ΔpetB strains contain deletions of the petA (encoding cytochrome f) and petB (encoding cytochrome b6) genes, respectively.

Sequence Analysis—Plasmid pcCS1–1 was digested with SacI and religated to make the plasmid pcCS1–4 in which the 5′-region of genomic DNA in pcCS1–1 was deleted. pcCS1–4 was used as a template for genomic sequencing. Nested deletions of plasmids containing cDNA fragments were made using the Erase-a-Base kit (Promega Corp., Madison, WI). Plasmids were sequenced using the Silver Sequence DNA sequencing system (Promega) or by dye terminator cycle sequencing using 3′ dye-labeled dideoxynucleotide triphosphates and run on an ABI PRISM 377 DNA sequencer (Perkin-Elmer). The DNA sequence was derived from sequencing the entire length of both strands of the cDNA and genomic clones.

RESULTS

Isolation of Cytochrome b6f-deficient Mutants—Nuclear transformation was used to generate insertion mutations in genes required for biogenesis of the cytochrome b6f complex of *C. reinhardtii*. Approximately 2500 independent transformants were grown on acetate-supplemented plates and subsequently screened for mutants that had lost the ability to grow phototrophically. Eight nonphotosynthetic transformants were identified and screened further for defects in accumulation of the cytochrome b6f complex by immunoblot analysis using an antibody against subunit IV (SuIV) to probe total cell extracts. Since strains that carry mutations affecting the accumulation of cytochrome f, cytochrome b6, or SuIV also fail to accumulate the other subunits of the complex (14, 37, 50), the abundance of SuIV is diagnostic of the entire cytochrome b6f complex. Four of the eight strains accumulated drastically reduced amounts of SuIV (strains 3, 9, 30, and 34; Fig. 1). These strains were designated as abf (accumulation of the b6f complex) mutants. The abf mutants did not accumulate cytochrome b6 or cytochrome f, as expected (Fig. 1). All abf mutants exhibited wild-type levels of the D2 protein of PS II, which suggests that these mutations do not affect the accumulation of cytochrome b559, which is an integral component of PS II.

We next performed a Southern blot analysis to determine whether the mutants exhibited an RFLP that might indicate whether the mutants exhibited an RFLP that might indicate whether the mutants exhibited an RFLP that might indicate a defect in either PetC or PetM. The two known nuclear genes that encode subunits of the complex. Since no PetC or PetM RFLPs were observed (data not shown), we concluded that the lesions in the abf mutants most likely affected 1) a previously unidentified nucleus-encoded subunit of the complex, 2) a regulatory gene required for the expression of structural genes of the complex, or 3) a gene required for the maturation or assembly of the complex.

Strain abf3 Does Not Accumulate Cytochrome c6—Nuclear mutations have defined four loci, CCS1–CCS4, involved specifically in the biogenesis of chloroplast c-type cytochromes. These strains exhibit a pleiotropic c-type cytochrome deficiency that is characteristic of a defect at the post-translational step of heme attachment. To determine whether any of the abf mutants harbored lesions of this type, the mutants were screened for cytochrome c6 accumulation by performing immunoblots, which assess polypeptide abundance, and heme staining, which assesses holocytochrome abundance. Cell extracts were isolated from cells grown in copper-deficient medium to induce transcription of *Cyc6*, which is repressed by copper (27, 51). The B6 mutant of *C. reinhardtii*, which contains a mutation in the chloroplast ccsA gene and does not accumulate cytochrome f or cytochrome c6 (29), was included as a control. As shown in Fig. 2, A and B, holocytochromes c6 and f do not accumulate in either abf3 or B6. To confirm that the cells were copper-deficient and hence under conditions allowing expression of the *Cyc6* gene, the extracts were tested for plastocyanin content, since cells accumulate plastocyanin only when the medium contains copper. Since little plastocyanin was detected under copper-deficient conditions (Fig. 2B, bottom; compare lanes 2, 4, and 6 with lanes 1, 3, and 5), the cells were indeed copper-deficient. Moreover, *Cyc6* transcripts accumulated under these conditions (Fig. 2C). Hence, the cytochrome c6 deficiency could...
clones isolated from a accumulation of petA contamination in the medium. The mutation did not affect by selection for photoautotrophic growth. The blots were exposed to a Molecular Dynamics PhosphorImager screen as follows: The blots were hybridized with a Ccs1-specific probe (Fig. 3, lane 2), and abf3 isolated from wild type cells (lane 1), abf3 (lane 2), and abf3 complemented by clone AIII-4 (lane 3). The blot was hybridized with a Ccs1-specific probe (probe 3, Fig. 4). B, relative positions of Ccs1-hybridizing clones isolated from a λ genomic library. C, N, S, and St indicate restriction sites for ClaI, NotI, SalI, and Stul, respectively (see Fig. 4 for a complete map of the locus). The stippled box represents the fragment of Chlamydomonas genomic DNA recovered by plasmid rescue, which flanks the pNIT1 insertion in abf3. An 8-kb NotI fragment from AIII-4 was subcloned into pBluescript KSII* to generate the plasmid pCcs1–1. pCcs1–2 consists of the 6.5-kb SalI/Stul fragment of pCcs1–1 cloned into pBluescript KSII*. Complementation experiments involved glass bead-mediated transformation of abf3 with indicated DNA fragments, followed by selection for phototrophic growth. + indicates that phototrophic transformants were recovered. C and D, accumulation of cytochrome (Cyt) f and cytochrome c6 in complemented strains. Proteins from the insoluble membrane fraction (C) or soluble fraction from either copper-free (−) or copper-supplemented (+) cell cultures (D) were analyzed for accumulation of cytochrome f(C) and cytochrome c6(D) by immunoblot analysis and heme staining.

FIG. 3, Complementation of abf3. A, Southern blot of SalI-digested total DNA isolated from wild-type cells (lane 1), abf3 (lane 2), and abf3 complemented by clone AIII-4 (lane 3). The blot was hybridized with a Ccs1-specific probe (probe 3, Fig. 4). B, relative positions of Ccs1-hybridizing clones isolated from a λ genomic library. C, N, S, and St indicate restriction sites for ClaI, NotI, SalI, and StuI, respectively (see Fig. 4 for a complete map of the locus). The stippled box represents the fragment of Chlamydomonas genomic DNA recovered by plasmid rescue, which flanks the pNIT1 insertion in abf3. An 8-kb NotI fragment from AIII-4 was subcloned into pBluescript KSII* to generate the plasmid pCcs1–1. pCcs1–2 consists of the 6.5-kb SalI/StuI fragment of pCcs1–1 cloned into pBluescript KSII*. Complementation experiments involved glass bead-mediated transformation of abf3 with indicated DNA fragments, followed by selection for phototrophic growth. + indicates that phototrophic transformants were recovered. C and D, accumulation of cytochrome (Cyt) f and cytochrome c6 in complemented strains. Proteins from the insoluble membrane fraction (C) or soluble fraction from either copper-free (−) or copper-supplemented (+) cell cultures (D) were analyzed for accumulation of cytochrome f(C) and cytochrome c6(D) by immunoblot analysis and heme staining.

not result simply from lack of Cyc6 expression due to copper contamination in the medium. The mutation did not affect accumulation of petA mRNA (Fig. 2C), and pulse-chase labeling experiments demonstrated that abf3, like B6 and previously described heme attachment mutants (11), synthesizes but is unable to accumulate cytochrome f (Fig. 2D). The presence of plastocyanin and the PS II-associated Oee1 protein (data not shown) in extracts of cells grown in the presence of copper confirms that the synthesis of other luminal proteins is not affected in these strains. Thus, it is unlikely that abf3 is defective in some component of the chloroplast-SecA thylakoid transport pathway (52, 53). Together, these data suggest that abf3 most likely harbors a mutation(s) in a gene(s) required for the attachment of heme to chloroplast c-type cytochromes.

The Ccs1 Gene Complements the Cytochrome b6f Complex and Cytochrome c6 Deficiencies in abf3—A Southern blot analysis of DNA isolated from abf3 that was hybridized with a Nil1-specific probe revealed that the mutant harbored a single insertion of pNIT1 (data not shown). Since the insertion in abf3 was presumably a consequence of this plasmid DNA insertion, we attempted plasmid rescue to isolate the DNA that flanked the insertion site in abf3 (47). The recovered plasmid contained a 1.3-kb C. reinhardtii DNA fragment. When a genomic DNA blot was probed with this fragment, an RFLP was detected between wild type and abf3 (Fig. 3A, lane 1 and 2). If the nonphotosynthetic phenotype was indeed due to the insertion, the RFLP should cosegregate with the mutant phenotype in genetic crosses. However, crosses between abf3 and an appropriate marker strain did not yield viable progeny. Therefore, a molecular genetic approach was pursued. To isolate a wild-type genomic sequence corresponding to the rescued DNA fragment, we screened a bacteriophage λ genomic library with the rescued DNA fragment. Of the six positive clones, two (λIII-4 and λIII-7) contained DNA that complemented the photosynthetic defect in abf3 (Fig. 3B). Southern blot analysis of DNA isolated from wild type cells, abf3, and a transformant rescued by one of the λ clones (abf3::λIII-4) provided molecular evidence of complementation; abf3::λIII-4 contains both the introduced wild-type
gene as well as the RFLP due to the insertion (Fig. 3A, lane 3). Subsequent subcloning and transformation experiments delimited the complementing region to a 6.5-kb DNA fragment (pCcs1-2 in Fig. 3B). Control transformation experiments with no DNA did not yield spontaneous photosynthetic revertants of abf3. The phototrophic transformants accumulated cytochrome f, as assessed by immunoblots and heme staining (Fig. 3C), and as expected, the complemented strains also accumulated wild-type levels of both cytochrome b₆ and SuIV (data not shown).

Since selection for phototrophic growth relies only on restoration of cytochrome f function, complemented transformants were also tested for cytochrome c₆ accumulation by immunoblot and heme stain analyses. As shown (Fig. 3D), abf3 strains that had been complemented with either lIII-4 or plasmid pCcs1-1 also accumulated wild-type levels of cytochrome c₆. These results demonstrated that a single nuclear mutation resulted in the deficiencies of the cytochrome b₆f complex and cytochrome c₆ in abf3. We conclude that the product of this nuclear gene is essential for the biogenesis of both chloroplast c-type cytochromes. The gene has been designated Ccs1 (c-type cytochrome synthesis).

Ccs1 Shares Significant Similarity to the Ycf44 Family—The primary sequence of the protein potentially encoded by the Ccs1 gene was determined by sequencing a cDNA clone isolated from a λ ZAP library. We screened 83104 plaques and identified a single positive plaque, which was significantly smaller than the other plaques. Although the λ ZAP vector system was designed to permit the direct recovery of plasmids containing cDNA inserts, we were unable to accomplish this with Ccs1. Therefore, the cDNA insert was obtained by amplification of the phage DNA using vector-specific primers and polymerase chain reaction. Attempts to clone the amplified insert into a plasmid vector also proved unsuccessful. The small size of the positive plaque and our inability to recover a cDNA-containing plasmid suggested that the cDNA might be expressed and toxic to E. coli. These problems were circumvented by subcloning the polymerase chain reaction-amplified cDNA insert as two separate fragments. The resultant plasmids were used as templates to determine the nucleotide sequence of the cDNA. The cDNA sequence was confirmed by determining the sequence of the

![Fig. 5. Nucleotide sequence and deduced amino acid sequence of Ccs1 cDNA. Underlined nucleotides indicate an A-rich region preceding the initiation codon. The 3' terminus of the cDNA contains a 28-bp polyA tail. The black arrowhead indicates the site of the pNIT1 insertion in abf3. GenBank™ accession numbers are U70999 (genomic sequence) and U71000 (cDNA). The numbers indicate amino acid residues; the open arrowhead indicates the putative transit peptide cleavage site; amino acid residues in italicized boldface type (residues 385–428) correspond to the alanine plus serine-rich region of Ccs1.](http://www.jbc.org/fig/137551a.png)
genomic DNA in pCcs1–3.

Alignment of the genomic and cDNA nucleotide sequences revealed that Ccs1 consists of 10 exons and 9 introns (Fig. 4A). Southern blot analysis revealed that Ccs1 is a single copy gene in C. reinhardtii (data not shown). We also determined the nucleotide sequence of the region spanning the site of the pNIT1 insertion in abf3 and found that the plasmid had inserted into the 10th exon of the gene, 70 bp from the termination codon (shown by an arrowhead in Fig. 4A). Southern blots revealed that the insertion in abf3 was accompanied by a deletion of approximately 200 bp of C. reinhardtii DNA upstream of the insertion site (data not shown). These alterations prevented the accumulation of Ccs1 transcripts (Fig. 4B).

The Ccs1 cDNA contains an ORF of 1839 nucleotides capable of encoding a 613-amino acid protein with a calculated molecular mass of 64.9 kDa (Fig. 5). The initiation codon is preceded by a short A-rich region (underlined sequence in Fig. 5) that is typical of many nuclear genes in C. reinhardtii (54–57) and vascular plants (58). 5’-Rapid amplification of cDNA ends of poly(A)+ RNA isolated from wild-type cells showed that the 5’-end of the mRNA contained approximately 120 additional nucleotides upstream of the 5’-end of the cDNA. However, an examination of the nucleotide sequence of this region did not reveal alternative initiation codons. A consensus polyadenylation addition site (TGTTAA) was not found in the 3’-untranslated region, despite the fact that the cDNA is intact as evidenced by the presence of a poly(A) tract at the 3’-end.

A search of the protein databases revealed five proteins of unknown function with significant similarity to Ccs1 (Fig. 6). Three of the proteins are ORFs encoded by ycf44 (hypothetical chloroplast frame) genes in oxygen-evolving photosynthetic organisms including the cyanobacterium Synechocystis strain PCC 6803 genome (33% identity; Ref. 59), the chloroplast genomes of the red alga Porphyra purpurea (26% identity; Ref. 60), and the brown alga Odontella sinensis (25% identity; Ref. 61). The fourth and fifth proteins, the resB gene product of Bacillus subtilis (62) and an ORF in the genome of Mycobacterium leprae, exhibit much less similarity to Ccs1 (11 and 12% similarities, respectively). Alignment of these proteins revealed two domains that are unique to Ccs1: an N-terminal extension, and an alanine plus serine-rich region in the central portion of the polypeptide (positions 385–428). Overall, the hydropathy profiles of Ccs1, ResB, and the Ycf44 proteins display remarkable similarity (Fig. 7). The N-terminal part of the extension resembles the stroma-targeting domains of chloroplast transit peptides, since it has a net positive charge, and the sequence VRC at positions 36–38 corresponds to the cleavage site ((V/I)X(A/C)) of proteins that are imported into chloroplast (63). However, there is an acidic residue and a tyrosine in this region, which are unusual in transit peptides. The remainder of the N-terminal extension resembles the stroma-targeting domains of chloroplast transit peptides, since it has a net positive charge, and the sequence ZRC at positions 36–38 corresponds to the cleavage site ((V/I)X(A/C)) of proteins that are imported into chloroplast (63).

3 K. Robinson, unpublished data (GenBank™ accession number U00018).

DISCUSSION

We have isolated a C. reinhardtii mutant, abf3, that is deficient in both the cytochrome b6f complex and cytochrome c6. Since an intact copy of the Ccs1 gene, which is disrupted in
abf3, complements both deficiencies, we conclude that Ccs1 is required for the biogenesis of both the cytochrome b6f complex and cytochrome c6. abf3 belongs to a class of previously described C. reinhardtii nuclear mutants defective in one of the final steps of chloroplast c-type cytochrome biosynthesis, e.g. heme transport across the thylakoid membrane, maintenance of protoheme and the apocytochrome heme-binding site in a reduced state, or covalent attachment of the heme prosthetic group to the apocytochrome (11). Mutations in these genes prevent formation of the holocytochromes and result in the accelerated turnover of the nonfunctional apocytochromes. Nei-

The lack of similarity between Ccs1 and gene products with known activities has precluded homology-based assignment of a specific biochemical function in the heme attachment pathway. However, the similarity of Ccs1 to the resB gene product of B. subtilis is consistent with our conclusion that Ccs1 does indeed encode a product involved in the biogenesis of c-type cytochromes. In B. subtilis, the resA, resB, and resC genes belong to the same transcriptional unit, suggesting that they function in a common pathway. The resA gene product is similar to protein disulfide isomerases and thioredoxin-like proteins, the latter of which have been shown to be required for the biogenesis of c-type cytochromes in Rhodobacter capsulatus and Bradyrhizobium japonicum (the helX and the ilpB gene, respectively; Refs. 64–66). Similarly, the resC gene product is related to CcsA and, like CcsA, contains a sequence motif that is found in a family of proteins that are also required for the biosynthesis of c-type cytochromes in bacteria (Ccl1/CycK in R. capsulatus/B. japonicum; Refs. 65 and 66). The presence of the resB gene within this operon is a strong indication that resB and its homologues, Ccs1 and ycf44, also encode proteins involved in the biogenesis of c-type cytochromes, and the phenotype of the abf3 strain confirms the function of one member of this family. Although the putative homologues of CcsA and Ccs1 in M. leprae have not been studied, they are organized in an operon as in B. subtilis.

It is very likely that the Ccs1 gene product is chloroplast-localized, because 1) it has an N-terminal extension relative to Ycf44 that might serve as a chloroplast import signal; 2) the biosynthesis of holocytochromes c6 and f share common steps only within the chloroplast; and 3) the homologous ycf44 gene products are encoded in the plastid genomes of the brown and red algae O. sinensis and P. purpurea, respectively (60, 61). The difference in the location of these related genes suggests that they were translocated from the plastid to the nuclear genome in C. reinhardtii. Based on their hydrophobic nature (Fig. 7), it is tempting to speculate that these genes encode membrane-associated proteins. We are currently generating antibodies against Ccs1 to permit the localization of the protein by subcellular fractionation and immunolocalization.

Although very little is known about the mechanisms involved in the biosynthesis of c-type cytochromes within the chloroplast, there are clearly similarities with the bacterial pathway. For instance, a common set of gene products participates in the ligation of heme to both membrane-bound and soluble c-type cytochromes in chloroplasts and bacteria (26, 64–66). This is in contrast to the situation in mitochondria, where two distinct cytochrome c heme lyases are required for the attachment of heme to the soluble cytochrome c and membrane-associated cytochrome c (reviewed in Ref. 67). Furthermore, while there appears to be no relationship between mitochondrial cytochrome c heme lyases and the products of cytochrome biogenesis genes in chloroplasts and bacteria, there are clear relationships between at least two sets of genes involved in the biogenesis of c-type cytochromes in chloroplasts and bacteria, specifically, between ccsA and cycK/ccl1 (29, 64) and between Ccs1/ycf44 and resB (this work).

In C. reinhardtii, there is at least one chloroplast locus (ccsA) and four nuclear loci (CCS1–CCS4) involved in the biogenesis of chloroplast c-type cytochromes. Clearly, interactions between these nucleus- and chloroplast-encoded gene products are involved in the process of c-type cytochrome biogenesis. Ultimately, we would like to understand the roles that these nucleus- and chloroplast-encoded proteins play in the biogenesis of chloroplast c-type cytochromes. The genetic approaches outlined in this paper, combined with biochemical analyses of

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the proteins and pathways, hold the promise of supplying answers to these questions.

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