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Complementation of a *Chlamydomonas reinhardtii* Mutant Defective in the Nuclear Gene Encoding the Chloroplast Coupling Factor 1 (CF_1) γ-Subunit (*atpC*)[1]

Eric J. Smart[2] and Bruce R. Selman[3]

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Chlamydomonas reinhardtii strain *atpC*1 is a mutant defective in the nuclear gene that encodes the CF_1 ATP synthase γ-subunit polypeptide. Photoautotrophic growth was restored to *atpC*1 after it was transformed with wild-type DNA. Transformed strains were acetate-independent and arsenate-sensitive, similar in phenotype to the progenitor wild-type strain from which *atpC*1 was generated. Three transformed strains were examined in detail. Southern blot analyses demonstrated that the transformants were complements and not revertants. The transforming DNA integrated into the nuclear genome in a nonhomologous manner and at a low copy number. Northern blot analyses showed that the γ-subunit mRNA in the complemented strains was expressed at the same relative level as that of wild-type. Western blots of total protein showed that whereas *atpC*1 was unable to synthesize any CF_1 γ-subunit, all three complemented strains could. Furthermore, the Western blot analyses demonstrated that the mutation in *atpC*1 had a pleiotropic effect on the accumulation of the CF_1 β-subunit which was relieved upon complementation. Cell extracts from *atpC*1 did not have any CF_1-dependent catalytic activity, whereas extracts from all of the complemented strains and the wild-type strain had identical activities.

KEY WORDS: Coupling factor; Chlamydomonas; gamma; ATPase; mutant; complementation.

INTRODUCTION

The chloroplast energy transducing ATP synthase belongs to the family of F_0-F_1 type coupling factor complexes. In the chloroplast, this enzyme (CF_0-CF_1)[4] utilizes the protonmotive force developed across the thylakoid membrane to synthesize ATP (Merchant and Selman, 1985). CF_0 is the intrinsic membrane sector and is composed of four unique polypeptides (designated I to IV) (Schneider and Altendorf, 1987). It forms a proton-specific channel (Schneider and Altendorf, 1987) that is linked to the extrinsic membrane sector, CF_1. CF_1 contains the catalytic sites for ATP synthesis and hydroysis (Merchant and Selman, 1985; Strotmann and Bickel-Sandkotter, 1984) and is compsed of five unique polypeptides (designated α to ϵ) with a rigorously conserved stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Merchant and Selman, 1985). Although the mechanism of catalysis is poorly understood, all F_0-F_1 type ATP synthases probably function similarly (Strotmann and Bickel-Sandkotter, 1984).

A great deal of time and effort has been expended in examining the functions of individual subunits of

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[2] Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235-9053.

[3] Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706.

[4] CF_1, chloroplast coupling factor 1; CF_0, chloroplast coupling factor 0; *E. coli, Escherichia coli*; PS3, thermophilic bacterium PS3; TAP, Tris-acetate-phosphate; TP, Tris-phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PCR, polymerase chain reaction.

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the CF_1 ATP synthase. This analysis has led to the suggestion that the active sites for catalysis are located on the β-subunits (Bruist and Hammes, 1981), the γ-subunit serves as a "proton gate" (Ketcham *et al.*, 1984), the δ-subunit functions in the productive binding of CF_1 to CF_0 (Andreo *et al.*, 1982), and the ϵ-subunit prevents the nonproductive hydrolysis of ATP, i.e., it is an ATPase inhibitor (Richter *et al.*, 1984). Whereas all of these activities of the CF_1 polypeptides are mimicked by peptides in complexes associated with other membrane systems, the CF_1 γ-subunit seems to be additionally involved in a type of regulation that is unique to the choroplast enzyme, namely the "redox control" of catalytic activity (Ketcham *et al.*, 1984).

Our knowledge concerning the redox regulation of the chloroplast enzyme has come mostly from *in vitro* studies with the spinach enzyme. The spinach CF_1 γ-subunit has four cystinyl residues, two of which are part of a cystinyl intrapeptide disulfide bridge (Moroney *et al.*, 1984). It is this bridge that appears to be involved in redox regulation (Ketcham *et al.*, 1984). Although the oxidized enzyme can catalyze high rates of ATP synthesis when provided with a large protonmotive force, reduction of the disulfide bridge to vicinal dithiols reduces the magnitude of the protonmotive force necessary to drive maximal rates of phosphorylation (Mills and Mitchell, 1984) and increases the apparent quantum yield for phosphorylation (Mills and Mitchell, 1984). Furthermore, the reduced enzyme retains catalytic activity as an ATPase in the absence of a protonmotive force (the so-called "light-triggered" ATPase) until either the source of ATP is exhausted (Strotmann *et al.*, 1987) or the enzyme is re-oxidized (Mills and Mitchell, 1984). The observation that the illumination of leaves (Morita *et al.*, 1982) and algae (Selman-Reimer and Selman, 1988) prior to the isolation of CF_1 results in an activated ATPase that can be inhibited by oxidants suggests that a similar type of redox control occurs *in vivo* as well.

Most of the conclusions about the role of the CF_1 γ-subunit in redox regulation have come from either chemical modification or partial reconstitution experiments. Unfortunately, in contrast to the analogous ATP synthase complexes associated with the plasma membranes of the prokaryotes *Escherichia coli* and PS3 (Vogel and Steinhart, 1976; Yoshida *et al.*, 1977), it has not yet been possible to reconstitute a functionally active CF_1 from its constituent subunits. One alternative approach to chemical modification is to genetically modify the subunits and to examine the effects of these modifications on the catalytic activities of the enzymes. To that end, we have been developing a genetic system that will allow us to manipulate the primary structure of the *Chlamydomonas reinhardtii* CF_1 γ-subunit *in vivo*.

The primary advantage of using *C. reinhardtii* as a model system is its ability to grow exclusively on acetate (Harris, 1989). The capability of *C. reinhardtii* to grow heterotrophically allows one to selectively destroy components of the photosynthetic apparatus and to maintain the cells as conditional lethal strains. We previously exploited this property of *C. reinhardtii* to generate a strain that has a defect in the 5′ half of the nuclear gene that encodes the CF_1 γ-subunit (the *atpC* gene) (Smart and Selman, 1991a). This strain, designated *atpC* 1, does not accumulate any mRNA encoding the CF_1 γ-subunit nor any γ-subunit polypeptide (Smart and Selman, 1991a). Strain *atpC*1 is an absolute acetate auxotroph and is capable of growing in the presence of a high (2.5 mM) concentration of arsenate. The mutation in *atpC*1 is "tight" for we have never observed a spontaneous reversion of the phenotype.

In this work we have used *atpC*1 as the recipient strain for transformation with a 4.3-kb fragment of nuclear DNA that contains the promoter as well as the transcription unit for the CF_1 γ-subunit gene. We demonstrate high efficiency, nonhomologous transformation that results in genetic complementation and restoration of the wild-type phenotype. Three complemented strains are characterized in detail with respect to their total DNA, mRNA, and gene products.

MATERIALS AND METHODS

Strains and Culture Conditions

The *Chlamydomonas reinhardtii* strain *nit*1-305 (cw 15, mt^-) was kindly provided by P. Lefebvre (University of Minnesota) and was the ATP synthase wild-type progenitor used in this study. The mutant strain *atpC*1 was generated from *nit*1-305 as previously described (Smart and Selman, 1991a). Both strains were maintained under constant illumination on Tris-acetate-phosphate (TAP) plates containing 1.5% agar (Harris, 1989). Cells were cultured in liquid TAP in 50- to 2,000-ml flasks with constant stirring.

Isolation, Subcloning, and Sequencing of the CF_1 γ-Subunit Gene Promoter

An EMBL-3 λ phage library containing *C. reinhardtii* nuclear DNA was the generous gift of Dr. M. Goldschmidt-Clermont (1986). The library was screened as previously described (Sambrook *et al.*, 1989). Nick-translated CF_1 γ-subunit *c*DNA probes containing [α-^{32}P]dCTP (DuPont) were hybridized to nylon filters containing λ DNA for 16 h at 70°C. The filters were washed four times at 70°C with 15 mM NaCl and 0.5% (w/v) sodium dodecyl sulfate for 1 h each. Hybridized plaques were visualized by autoradiography, purified, and λ DNA isolated (Sambrook *et al.*, 1989). A *Sal* I restriction fragment, designated Pγ, from one of the λ plaques was cloned into the plasmid pBluescript II SK$^+$ (Stratagene, La Jolla, California). This plasmid was amplified in XL1-Blue cells (Stratagene) and isolated by using a Qiagen anion-exchange column (Qiagen, Studio City, California). All of the information upstream of the apparent start site of transcription was sequenced by the standard dideoxy method using a Sequenase kit (United States Biochemical, Cleveland, Ohio).

Transformation and Selection

C. reinhardtii cells were transformed by the polyethylene glycol/glass bead method as previously described (Kindle, 1990). Briefly, *atpC*1 cells were grown in liquid TAP to a density of approximately 10^6 cells per ml and concentrated to 8×10^6 cells per ml by centrifugation at $3,000 \times g$ for 2 min. The cells were resuspended and washed in 15 ml of TP [Tris (0.2 mM)-phosphate (0.69 mM); pH 7.0] buffer, collected by centrifugation and resuspended in 0.5 ml of TP buffer. One-half milliliter of acid-washed glass beads (0.5 mm in diameter) was added to the suspension. Filter-sterile polyethylene glycol was added to a final concentration of 12% (v/v) along with the transforming DNA. The transforming DNA was either 2 μg of *Sal* I restricted plasmid containing Pγ, 2 μg of *Sal* I restricted SK$^+$, 2 μg of sonicated Herring sperm DNA, or no DNA. The contents of the tube were mixed for 15 s at full speed on a Vortex Genie. After the beads settled, 50-μl aliquots were spread onto TP-agarose plates (1.5%), and the plates were placed in constant bright light for 1–3 weeks. Transformants were maintained on TP-agarose plates under constant bright light.

TP-agarose was prepared by suspending agarose (1.5%) in 4 liters of double-distilled water and stirring overnight at 4°C to remove trace amounts of acetate. The water was removed and fresh water added four times. The agarose suspension was then equilibrated with a TP buffer by removing the water and adding 4 liters of TP and stirring overnight at 4°C. This washing procedure was repeated three more times. The medium was then autoclaved and used immediately.

DNA and RNA isolation

C. reinhardtii total DNA was isolated by the sodium sarcosyl lysis method (Harris, 1989) with the modifications previously described (Smart and Selman, 1991a). Total RNA was isolated by LiCl precipitation as previously described (Smart and Selman, 1991a).

PCR Analysis

Polymerase chain reaction samples contained 1 μg of total DNA, 500 ng of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01% (w/v) gelatin, 0.1% (v/v) Triton X-100, 2.5% (v/v) dimethyl sulfoxide, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, Wisconsin) in a total volume of 50 μl. Reaction conditions were: 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 5 min, 55°C for 2 min, and 72°C for 4 min; 4°C end.

Southern and Northern Blot Analyses

Southern and Northern blot analyses were performed as previously described (Sambrook *et al.*, 1989). The nucleic acid was separated by electrophoresis and transferred to a charge-modified polysulfone binding matrix (Gelman Sciences, Ann Arbor, Michigan). The nucleic acid was fixed by heating at 65°C for 2 h. Nick-translated *c*DNA probes containing [α-^{32}P]dCTP were hybridized to the DNA-containing membranes for 16 h at 65°C. The membranes were washed three times at 65°C with 15 mM NaCl and 0.5% sodium dodecyl sulfate for 45 min each. Hybridized bands were visualized by autoradiography.

Immunoblot Analysis

Crude protein fractions were isolated from 50 ml cell cultures by chloroform extraction as previously described (Smart and Selman, 1991a). Protein concentrations were determined according to standard methods (Sambrook *et al.*, 1989), using bovine serum

albumin as a standard. Approximately 100 μg of the protein samples were electrophoresed in a denaturing sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) essentially as described previously (Sambrook *et al.*, 1989). After electrophoresis, gels were either stained with Coomassie brilliant blue to visualize polypeptide bands, or transblotted.

Proteins were transblotted onto nitrocellulose membranes (3 h at 4°C, 70 v) essentially as previously described (Sambrook *et al.*, 1989). Unoccupied protein binding sites were blocked by incubating the membranes for 4 h at room temperture in buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 M NaCl (TBS), and 3% (w/v) gelatin. The primary antibody (rabbit anti-*C. reinhardtii* CF_1 γ-subunit or chicken anti-*Dunaliella salina* CF_1 β-subunit) preparations were diluted 1/1000 in TBS containing 1% (w/v) gelatin and 0.01% (w/v) NaN_3 and incubated with the membranes for 16 h at room temperature. The secondary antibody was either horseradish peroxidase conjugated rabbit anti-chicken IgG (Pel Freez, Rogers, Arkansas) or horseradish peroxidase conjugated goat anti-rabbit IgG (BioRad, Richmond, California). Protein bands were visualized according to the manufacturer's instructions (BioRad, Richmond, California).

ATPase Assay

Protein samples enriched in CF_1 were isolated from 2 liters of cells as described previously (Selman-Reimer *et al.*, 1981). The cells were broken by sonication, and thylakoid-enriched membranes were pelleted by centrifugation at $2000 \times g$ for 1 min. These membranes were washed three times with 10 mM sodium pyrophosphate (pH 7.8) in order to remove contaminating ribulose bisphosphate carboxylase. Thylakoid-associated proteins were extracted from chloroform-disrupted membranes into a solution containing 20 mM Tricine-NaOH (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ATP, and 2 mM dithiothreitol (DTT). Remaining particulate matter was removed by centrifugation (39,100 $\times$ *g* for 10 min followed by 183,000 $\times$ *g* for 1 h). All protein fractions were prepared immediately prior to assay and were maintained at room temperature.

The catalytic activity of CF_1 was measured as a Mg^{2+}-dependent ATPase as previously described (Selman-Reimer *et al.*, 1981). When included in the assay, the final concentration of ethanol was 20% (v/v). Reaction mixtures, in 0.1 ml total volume, were incubated at 37°C for 2 min, and the hydrolysis of [γ-^{32}P] ATP (DuPont, Richmond, California) was determined as described (Selman-Reimer *et al.*, 1981). Antibody samples were prepared from chicken egg yolks (Polson *et al.*, 1980) either prior to (preimmune) or after (immune) inoculation with *Dunaliella salina* CF_1. The immune preparation used in this study has previously been demonstrated to cross react with *C. reinhardtii* CF_1 and to completely inhibit the ATPase activity (Selman-Reimer and Selman, 1986). In the experiment reported here, both immune and preimmune samples were used at a final concentration of 330 μg protein/0.1 ml of reaction mixture. All measurements were made in triplicate.

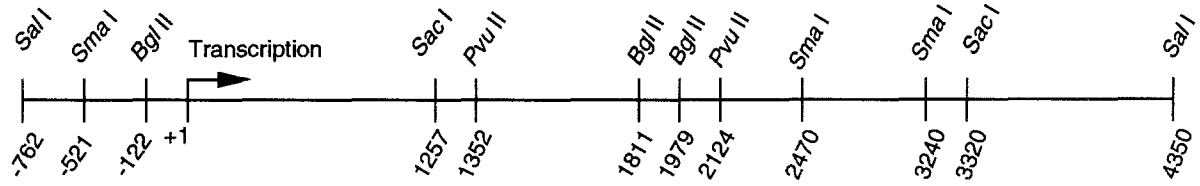
RESULTS

Isolation of the Promoter and Coding Regions for the CF_1 γ-Subunit Gene

C. reinhardtii strain *atpC*1 has a lesion in the nuclear gene that encodes the CF_1 γ-subunit (*atpC*) (Smart and Selman, 1991a). Before this strain can be used to study modifications of the CF_1 γ-subunit, it is necessary to demonstrate that wild-type ATP synthase activity can be restored to transformed cells. In principle, this can be accomplished by either correcting the mutation by homologous recombination or complementing the mutation by nonhomologous recombintion with a functional gene. Whereas homologous recombination into the chloroplastic genome is possible (Boynton *et al.*, 1988), it has not yet been observed for the nuclear genome (Kindle *et al.*, 1989). Consequently, we relied on complementation with the constraint that the transforming DNA must contain all of the information required for gene expression. Although we have isolated both the *c*DNA (Yu *et al.*, 1988; Yu and Selman, 1988) and the genomic DNA (Smart and Selman, 1991b) encoding the CF_1 γ-subunit, both lack a functional promoter and neither could be used for complementation experiments. We therefore attempted to isolate a fragment of genomic DNA that contained both a functional promoter as well as the transcription unit for the CF_1 γ-subunit gene.

We screened an EMBL-3 λ phage library containing *C. reinhardtii* DNA fragments (Goldschmidt –Clermont, 1986) and identified seven positive plaques. When the *C. reinhardtii* inserts were removed

A



B

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GTCGACGGACTTCAGGCGGACGGCGTGCGTACAAAGCTAT
CTAGCCATATACGCCTCCTCCATCTCCCCCGCCGCCTGGC
TGCACAGCAGGTGCTGCGGCAGCGCCCGGCGCTTTCAGCA
AGGCAGCGAGGCAGCGCAACGCCGCGCAGTTACCGCGCAG
CCTCTTCCTGCACCCACCCCCACAACAACGGACGGGAGCA
ACCCAGCCTCTGAAACCTGCTCCACACACGCGGTTGGGCC
CGGGGGGTGGCTCGTCGAGGGCTGGTGGGTAGCGACGGGT
AGCGACGGCTCGTCTCCGCCTTGCGCGCCCATTCGTCTTC
GTCCGACAGCCGACTGCACTTGAGGCGACATCCTCTGCTT
TTGCCTGTAATATGGGCCGTTCATACAGCACATTTTGGCA
ATCGCCCCAGTGTTGGGCGACCCTCACCGCACGCGCGCGA
AACATCGGCGCAACCTGATTGGCAATGGTGTCGCCTTACA
TCAAACCACTGCATGTAAACCTTAAATCCGAGGTCACGCG
GCCTCAGAGGACTCCCCGCAGCAGCGTTGCGCCACAATCT
CTCCTCGTGCAAGCTACTCCCAGGCTCCTGCATTCTATAA
GCGTAATTTTATGCCGGGTACGCTTGTGAATTGACGAAGA
TCTACTCGACGGTGTTCTGGTGAGCAAAATCGGAGGCAAA
CCCAATTGGCCCCCCTGGAGTGATAAGTCCTGGGTGCCAA
GTGCGCAAGTGAAGCCTTGAACTGCGCCTTTCCTTGCACC
TT-1

Fig. 1. Restriction map of Pγ and the nucleotide sequence for the promoter region of the nuclear gene encoding the *C. reinhardtii* CF_1 γ-subunit. (A) The putative start of transcription and the numbering system are based on our longest full length *c*DNA (Yu *et al.*, 1988). (B) The putative TATA and CAAT consensus sequences are underlined.

from the λ DNA by restriction with *Sal* I (Goldschmidt-Clermont, 1986), a 4350-bp fragment that hybridized to our *c*DNA probe was found in all seven isolates. This fragment was subsequently cloned into an SK^+ plasmid. Restriction analysis of this DNA fragment, which we have designated Pγ, showed that it contained the entire coding region of the CF_1 γ-subunit gene along with 762 bp of upstream and approximately 675 bp of downstream information. A restriction map of Pγ is shown in Fig. 1A.

Figure 1B shows the nucleotide sequence for the putative promoter region of Pγ. Because we have not yet established the actual transcriptional start site for the CF_1 γ-subunit gene, this numbering system is based on our longest full-length *c*DNA, with the first base in that *c*DNA being designated +1 (Yu and Selman, 1988). Upstream, at positions -163 and -212, there are consensus sequences for the TATA and CAAT boxes, respectively (Lewin, 1990). Although a consensus sequence for the GC box (GGGCCC) was not found, this is not surprising because eukaryotic promoters often lack one or more of the promoter elements. In order to determine if we had cloned enough upstream information for complementation, we transformed *atpC*1 cells with this DNA.

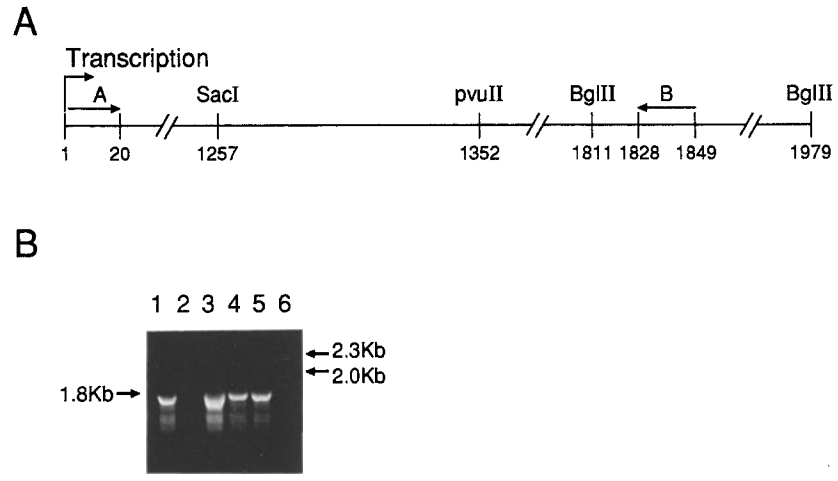

Fig. 2. PCR analysis of total DNA isolated from putative complemented strains. (A) Location of primers used to amplify the 5′ portion of the *atpC* gene. (B) The products were electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide. Lane: 1, wild-type; 2, *atpC*1; 3, PγC1; 4, PγC2; 5, PγC3; 6, λ *Hind* III standards.

Transformation of *atpC*1 and Selection for Potential Complements

Strain *atpC*1 is an absolute acetate auxotroph and is arsenate resistant (Smart and Selman, 1991a). In contrast, the wild-type strain, *nit*1-305, is photoautotrophic and arsenate sensitive. This provided us with both a strong selection for complementation, the demand that photoautotrophic growth be restored, and a test for complementation, the restoration of arsenate sensitivity.

Each set of transformation experiments included one sample containing Pγ (and plasmid) DNA and three negative controls. The four samples were (i) 2 μg of the SK^+ plasmid containing Pγ from which Pγ was excised by digestion of the plasmid with *Sal* I, (ii) 2 μg of SK^+ plasmid digested by *Sal* I, (iii) 2 μg of sonicated Herring sperm DNA, or (iv) no DNA. After treatment, the cells were spread onto TP-agarose media at a density of 4×10^5 cells per plate and grown at 27°C for 1–3 weeks under constant illumination. This selection forced cells to grow photoautotrophically.

Only cells that were transformed with the Pγ DNA fragment grew photoautotrophically (approximately 100 colonies per plate). Plates containing cells that were transformed with SK^+ plasmid DNA, Herring sperm DNA, or without DNA never produced colonies that could survive photoautotrophically. Furthermore, we should emphasize that a spontaneous reversion of the mutation in *atpC*1 has never been observed. Thus, these observations strongly suggest that it was the Pγ DNA fragement, and hence the wild-type CF_1 γ-subunit gene and promoter, that was responsible for the complementation of the defect in *atpC*1.

Fifty of the transformed photoautotrophic colonies were randomly selected and grown again on fresh TP-agarose plates. All 50 colonies remained viable. In order to determine if these colonies were arsenate resistant, they were then plated onto TAP-agar supplemented with 2.5 mM arsenate. All fifty complemented strains, as well as the wild-type strain, failed to grow, whereas the growth of *atpC*1 was unimpeded. These results further demonstrated that the Pγ DNA fragment was responsible for restoring a wild-type phenotype to *atpC*1 and that the CF_1 γ-subunit gene putative promoter was functional.

The efficiency of the Pγ DNA fragment to transform *atpC*1 and restore photoautotrophic growth was determined. A total of ten separate transformation experiments, each using 2 μg of Pγ DNA, were conducted as described above. On average, each plate contained about 100 photoautotrophically growing cells; however, the variability for any given plate was quite high. We estimate that the transformation efficiency was in the range of $1–2 \times 10^3$ cells per 2 μg of DNA.

PCR Analysis of Total DNA Isolated from Putative CF_1 γ-Subunit Gene Complemented Strains

Previously, we demonstrated that the lesion in strain *atpC*1 prevents polymerase chain reaction (PCR) amplification of the 5′ portion of the coding region for the *atpC* gene (Smart and Selman, 1991a). Figure 2 compares the ability of PCR to amplify the corresponding region in DNA isolated from wild-type (lane 1), *atpC*1 (lane 2), and three complemented strains (designated PγC1, PγC2, and PγC3; lanes 3 to 5). The predicted 1.8-kb product (Smart and

Selman, 1991a) was generated with DNA isolated from wild-type and all three complemented strains but not from *atpC*1. Clearly, the complemented strains contain a wild-type copy of the 5′ half of the CF_1 γ-subunit gene. [Note that an analysis of the 3′ half of the CF_1 γ-subunit gene would be meaningless because the mutation in strain *atpC*1 does not alter the 3′ half of the *atpC* gene (Smart and Selman, 1991a)].

Southern Blot Analyses

In order to distinguish between potential reversions and nonhomologous recombination leading to complementation, we examined the DNA from strains PγC1, PγC2, and PγC3 by a Southern blot analysis. Total DNA samples from wild-type, *atpC*1, and the three putative complemented strains were restricted with *Pvu* II and probed with radiolabelled CF_1 γ-subunit *c*DNA (Fig. 3). The DNA from wild-type contained three hybridizing fragments of sizes 2500, 1400 and 772 bp (lane 1). This was in agreement with our previous results (Smart and Selman, 1991a). Figure 1A shows that the 2500-bp band corresponded to the 5′ end of the gene, the 1400-bp band corresponded to the 3′ end of the gene, and the 772-bp band was an internal fragment (Fig. 1A). The DNA from *atpC*1 also produced the expected polymorphic banding pattern (lane 2) (Smart and Selman, 1991a). The 1400- and 772-bp bands remain unchanged, whereas the 2500-bp band disappeared and a 1300-bp band appeared. All three putative complemented strains contained the 772-, 1300- and 1400-bp bands characteristic of *atpC*1 but not the 2500-bp band found in the wild-type strain (lanes 3–5). The maintenance of the mutant polymorphic banding pattern in the DNA isolated from all three complemented strains clearly indicates that all three complemented strains have retained the mutated form of the CF_1 γ-subunit gene.

If the integration of the Pγ DNA fragment into the *atpC*1 genome was random, we would expect to see at least two additional polymorphic bands in the Southern blots of the complemented strains. These bands would correspond to the 5′ and 3′ ends of the Pγ DNA fragment. PγC1 and PγC3 contain at least two and maybe three additional bands (lanes 3 and 5). PγC2 (lane 4) contains at least five additional bands. More than two additional polymorphic bands in the complemented strains may have arisen as the result of either multiple integration events or rearrangements of the plasmid DNA prior to integration (Kindle *et al.*, 1989). Undigested total DNA samples were probed to confirm that the polymorphisms seen in the lanes from the complemented strains were due to integration and not an independently replicating plasmid. The undigested samples did not contain any plasmid bands (data not shown), indicating that the transforming DNA had integrated into the genome.

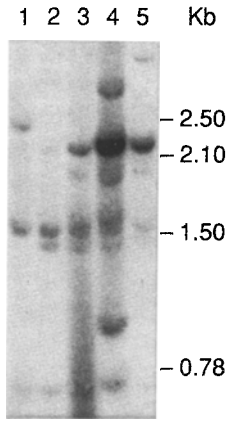

Fig. 3. Hybridization of nick-translated CF_1 γ-subunit encoding *c*DNA to *Pvu* II restricted DNA isolated from wild-type, *atpC*1, and complemented strains of *C. reinhardtii*. Total DNA was restricted for 15 h with *Pvu* II, electrophoresed in a 1% agarose gel, and transferred under alkaline conditons to a charge-modified polysulfone membrane. The membrane was blocked and hybridized with an [α-^{32}P]dCTP-labelled probe at 65°C. Lane: 1, wild-type; 2, *atpC*1; 3, PγC1; 4, PγC2; 5, PγC3.

SDS-PAGE and Western Blots of Crude Protein Isolated from Wild Type, *atpC*1, PγC1, PγC2, and PγC3

In order to further characterize the three complements, crude protein fractions were isolated and used for SDS-PAGE and Western blots. Protein samples from wild-type, mutant, and the complemented strains were electrophoresed in a 15% SDS-PAGE gel and stained with Coomassie blue. Figure 4A shows that all five samples contained similar polypeptides. When a similar gel was blotted onto nitrocellulose and probed with a rabbit anti-CF_1 γ-subunit serum, wild-type and all three complemented strains contained a cross-reacting band that corresponded exactly to the CF_1 γ-subunit polypeptide (Fig. 4C). However, no cross-reactive band was found in the lane containing crude protein isolated from *atpC*1. [Note that multiple cross-reactive bands are often observed in the region of the gel containing the CF_1 γ-subunit; (cf. Selman-Reimer and Selman, (1986)] and have been attributed to proteolytic degradation

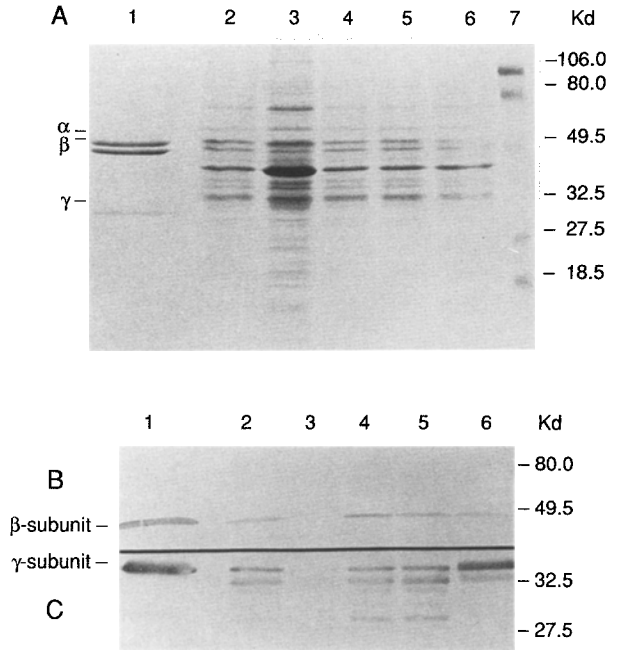

Fig. 4. SDS-polyacrylamide gel and Western blot analysis of crude protein isolated from wild-type, *atpC*1, PγC1, PγC2, and PγC3. (A) 100 μg of chloroform-extracted crude protein was electrophoresed in a 15% SDS-PAGE gel and stained with Coomassie blue. Identical protein samples were transferred to nitrocellulose membranes as detailed under Materials and Methods. (B) The membranes were blocked by incubating for 4 h at room temperature in buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 M NaCl (TBS), and 3% (w/v) gelatin. The primary antibody was chicken anti-*Dunaliella salina* CF_1β-subunit. (C) The primary antibody was rabbit anit-*C. reinhardtii* CF_1 γ-subunit. Lane: 1, purified CF_1; 2, wild-type; 3, *atpC*1; 4, PγC1; 5, PγC2; 6, PγC3.

of the CF_1 γ-subunit polypeptide. The observation that the lane containing the protein isolated from *atpC*1 failed to contain any of these bands would appear to confirm this supposition.] This Western blot confirms that the three complemented strains regained the ability to produce and accumulate an apparent wild-type CF_1 γ-subunit polypeptide.

Crude protein fractions isolated from the various strains were also blotted and probed with a chicken anti-CF_1 β-subunit antibody preparation. As above, protein from wild-type and all three complemented strains contained a polypeptide that cross-reacted at the position of the CF_1 β-subunit, whereas protein isolated from *atpC*1 was devoid of this polypeptide (Fig. 4B) even though *atpC*1 accumulated the CF_1 β-subunit mRNA (Smart and Selman, 1991a).

Northern Blot Analyses

Growth curves for the three complemented strains and the progenitor wild-type revealed no substantial differences in the culture doubling times (ca. 20 h; data not shown), suggesting that the expression of the CF_1 γ-subunit gene was not rate limiting for either heterotrophic or photoautotrophic growth. Nevertheless, it was of interest to compare the steady-state levels of CF_1 γ-subunit mRNA in each strain. For comparison, we also probed the total RNA isolated from each strain for the nuclear-encoded protein plastocyanin. These data are shown in Fig. 5. Qualitatively, no differences were seen in the amplitude of the signal corresponding to the CF_1 γ-subunit mRNA from wild-type and the complemented strains (lanes 1 and 3 to 5). Furthermore, the apparent size of the CF_1 γ-subunit mRNAs in the complemented strains was identical to that of wild-type, indicating that the RNAs were all processed properly. In contrast, RNA isolated from *atpC*1 failed to hybridize to the CF_1 γ-subunit *c*DNA probe (lane 2). On the other hand, RNA isolated from all the strains hybridized to the plastocyanin *c*DNA probe with approximately equal intensity. Quantitatively, the ratio of radioactive signal found in the CF_1 γ-subunit band to that in the plastocyanin band remained essentially constant for the wild-type and complemented strains (data not shown).

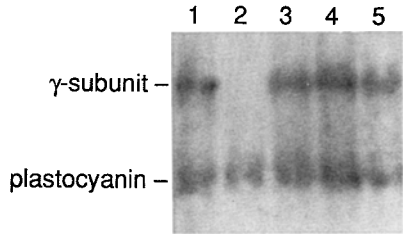

Fig. 5. Northern analysis of total RNA isolated from wild-type, *atpC*1, PγC1, PγC2, and PγC3. 1-μg samples of each RNA were denatured, electrophoresed in a 1% agarose gel, and blotted onto a polysulfone membrane. The membrane was blocked and hybridized at 65°C with nick-translated, labelled *c*DNA encoding the CF_1 γ-subunit and *c*DNA encoding plastocyanin. Lane: 1, wild-type; 2, *atpC*1; 3, PγC1; 4, PγC2; 5, PγC3.

ATPase Activities

In order to show that complementation restored enzymatic activity, we used partially purified protein fractions from wild-type, *atpC*1, and the three complemented strains to determine the rates of CF_1-catalyzed ATP hydrolysis. The data in Table I demonstrate that protein fractions from all of the strains has some basal ATPase activity, albeit the activity of the *atpC*1 preparation was substantially

Table I. Chloroplast Coupling Factor 1 ATPase Activity Associated with Protein Isolated from Several Strains of *C. reinhardtii* (μmol ATP hydolyzed/mg protein/min)

	Additions to reaction mixture			
Strain	None	Ethanol	Ethanol + preimmune	Ethanol + immune (anti-CF_1)
nit-305	7	34	32	3
*atpC*1	2	2	2	0
PγC1	7	32	35	2
PγC2	7	34	29	0
PγC3	7	35	35	0

[a] ATPase reactions were measured as previously described (Selman-Reimer *et al.*, 1981). Where noted, reaction mixtures contained 20% (v/v) ethanol and 330 μg of chicken egg yolk antibodies prepared either prior to (preimmune) or after (immune) immunization of a chicken with purified CF_1 isolated from *Dunaliella salina* (Selman-Reimer and Selman, 1986).

lower than those from wild-type and the complemented strains (2 vs. 7μmol ATP hydrolyzed/mg protein/min, respectively). The addition of ethanol (20% v/v) stimulated the basal rate about fourfold for protein preparations isolated from wild-type and complemented strains but had no effect on the activity of the *atpC*1 preparation. There were no notable differences in the CF_1-specific ATPase activities of the preparations isolated from the wild-type and complemented strains. The ethanol-stimulated activities from the wild-type strain and the complemented strains were completely inhibited by the monospecific antibody preparation directed against the *D. salina* CF_1 as was the very low ATPase activity associated with the protein preparation isolated from *atpC*1. We believe the latter to be an artifact, and we interpret these data as being consistent with the fact that *atpC*1 fails to make any functional CF_1.

DISCUSSION

We had previously generated the CF_1 γ-subunit deficient strain, *atpC*1, by nonhomologous integration of foreign DNA into the nuclear genome of *C. reinhardtii* (Smart and Selman, 1991a). The rationale for generating *atpC*1 was to utilize it as a tool for studying the redox regulation of the chloroplast ATP synthase. This strain would only be useful if wild-type enzymatic activity could be restored after a copy of the wild-type CF_1 γ-subunit gene was inserted back into the genome and expressed.

In order to complement *atpC*1, we had to first isolate the nuclear gene encoding the CF_1 γ-subunit. This DNA fragment had to contain enough upstream information to include a functional promoter that would drive the transcription of the CF_1 γ-subunit gene. From an EMBL-3 λ phage library, we isolated a 4350-bp DNA fragment, Pγ, that contained the entire transcription unit for the CF_1 γ-subunit plus 762 bp of upstream information. When the Pγ DNA fragment was used in transformation experiments, photoautotrophic colonies were obtained at a high efficiency, which strongly suggests that Pγ contains a functional promoter.

Because strain *atpC*1 is a stable, absolute acetate auxotroph, the selection that we used in our complementation experiments was extremely rigorous, namely the resotration of photoautotrophic growth. The frequency of transformation with Pγ DNA ranged between $1-2 \times 10^3$ cells per 2 μg of plasmid DNA. This efficiency of transformation is similar to that reported by Kindle (1990), who complemented a nitrate reductase mutation by transformation and nonhomologous integration. Our Southern blot analyses clearly demonstrated that the restoration of the wild-type phenotype in the strains that we examined was indeed due to complementation and not reversion.

All other ATP synthase mutants in *C. reinhardtii* that have been previously characterized have had lesions in the chloroplast-encoded subunits with the exception of a few nuclear mutation that effect the transcription and translation of the chloroplast *atp* genes (Piccioni *et al.*, 1981). Lemaire and Wollman, (1989) have characterized deletion mutants for the β- and ϵ-subunits of CF_1 as well as subunits I and IV of CF_0. In addition, frameshift mutations for the ϵ-subunit (Robertson *et al.*, 1990) are available. Notably, a lesion that prevents the expression of one of the ATP synthase subunits results in the absence of all of the others, probably because they are rapidly turned over

when they cannot assemble into mature complexes. This pleiotropic phenomenon also occurs with *atpC*1. In particular, even though the mRNA for the CF_1 β-subunit is expressed in *atpC*1 at wild-type levels (Smart and Selman, 1991a), the CF_1 β-subunit polypeptide is not detectable in protein extracts from the mutant (Fig. 4). This is probably the case for the other polypeptides as well.

In order for strain *atpC*1 to be useful in studying the redox regulation of the ATP synthase, transformation with a wild-type CF_1 γ-subunit gene must restore wild-type ATP synthase activity to complemented strains. A protein fraction isolated from thylakoid-enriched membranes from the mutant strain *atpC*1 does not have CF_1 ATPase activity (Table I). However, protein fractions isolated from all of the complemented strains do, and their specific activities are indistinguishable from the progenitor wild-type control. These data clearly demonstrate that complementation restores wild-type levels of enzymatic activity.

Complementation of the mutation in *atpC*1 demonstrates the usefulness of this strain in studying ATP synthase regulation. The mutation is stable as revertants have not been detected. In addition, a positive selection is available for the presence (arsenate sensitivity) and absence (acetate auxotrophy) of a functional ATP synthase. The versatility of this system is enhanced by the ability to restore enzymatic activity with a relatively small (ca. 4350 bp) and completely defined DNA sequence. This will make site-directed mutagenesis of the CF_1 γ-subunit gene straightforward and allow questions concerning the regulation and biogenesis of ATP synthase to be addressed. These studies are currently in progress.

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