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Intracellular Carbonic Anhydrase Is Essential to Photosynthesis in *Chlamydomonas reinhardtii* at Atmospheric Levels of CO_2[1]

Demonstration via Genomic Complementation of the High-CO_2-Requiring Mutant *ca-1*

Roel P. Funke, Joy L. Kovar, and Donald P. Weeks*

Department of Biochemistry, Center for Biotechnology, University of Nebraska, Lincoln, Nebraska 68588–0665

Genomic complementation of the high-CO_2-requiring mutant *ca*-1–12–1C of *Chlamydomonas reinhardtii* was achieved by transformation with DNA pools from an indexed cosmid library of wild-type genomic DNA. Transformation of mutant cells with cosmid DNA from two microtiter plates in the library produced colonies that grew phototrophically at atmospheric CO_2 levels. Transformations with cosmid DNA from each of the rows and files of the two plates pinpointed one well in each plate with a cosmid bearing the targeted gene. Sequencing of cosmid subclones revealed a gene encoding a recently identified *C. reinhardtii* chloroplast carbonic anhydrase (CAH3). Transformations with chimeric constructs combining different portions of the wild-type and mutant genes indicated the presence of a mutation in the 5′-half of the gene. Comparison of mutant and wild-type gene sequences in this region revealed a G-to-A substitution in the mutant gene, which produced a nonsense codon. The data presented demonstrate that the carbonic anhydrase produced from the *CAH3* gene is essential to the inorganic carbon-concentrating mechanism in *C. reinhardtii* and that genomic complementation can be a facile and efficient means for isolating genes associated with defects affecting photosynthesis and other physiological processes in this eukaryotic green alga.

Many cyanobacteria, green algae, and aquatic macrophytes have evolved CCMs that allow them to concentrate C_i and flourish in a CO_2-limited environment (for reviews, see Raven, 1985; Badger, 1987; Kaplan et al., 1990; Badger and Price, 1994). The CCM functions to increase internal CO_2 concentrations in the vicinity of the carboxylating enzyme Rubisco to levels that allow the enzyme to operate at or near maximal rates.

Although the existence of an active C_i uptake mechanism in many aquatic photosynthetic organisms has been known for many years, none of the essential components has been isolated in eukaryotic green algae. A C_i transporter (also referred to as a CO_2 pump) and CA, a zinc-containing enzyme that catalyzes the interconversion of CO_2 and HCO_3^- (Fukuzawa et al., 1992; Badger and Price, 1994), are thought to be key elements in the operation of the CCM (Badger, 1987). There are likely to be other structural components of the CCM, as well as gene products, that are needed to regulate acclimation of the cell to variable levels of CO_2.

Evidence for the potential involvement of an intracellular CA in the efficient utilization of C_i in green algae comes from the analysis of high-CO_2-requiring mutants that appear to be deficient in intracellular CA activity (Spalding et al., 1983a; Moroney et al., 1986). Mutants of *Chlamydomonas reinhardtii* with defects in their ability to accumulate C_i were isolated based on their ability to grow photoautotrophically in high (5%) CO_2 but not under atmospheric (0.03%) CO_2 (Spalding et al., 1983a, 1983b). Mutant *ca*-1–12–1C, the first high-CO_2-requiring mutant to be isolated (Spalding et al., 1983a), appeared to be deficient in intracellular CA activity. Sültemeyer et al. (1995) found that a soluble chloroplast CA appeared to be completely absent in this mutant, whereas an insoluble CA species was present at a much reduced level compared with wild-type cells. Neither of these enzyme forms was induced (derepressed) to generate increased CA levels when *ca*-1 cells were transferred from 5 to 0.03% CO_2. This is in sharp contrast to the marked increase in CA activity noted when wild-type cells are switched from high to low concentrations of CO_2 (Badger and Price, 1994; Sültemeyer et al., 1995). The phenotype of *ca*-1 could be mimicked in wild-type cells by the application of the CA inhibitor ethoxyzolamide (Spalding et al., 1983a). These data suggested that the gene that was defective in mutant *ca*-1 either encoded an essential chloroplastic CA or controlled the synthesis of one or more such enzymes in *C. reinhardtii*.

Progress toward understanding the roles of CA in the C_i-concentrating mechanism of *C. reinhardtii* can be made by the isolation and characterization of the enzyme itself (Carlson, 1995; Karlsson et al., 1995) or, more directly, by attempts to isolate genes responsible for defects in the assembly and/or synthesis of CCM components from mu-

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* Corresponding author; e-mail btec001@unlvm.unl.edu; fax 1–402–472–3139.

Abbreviations: CA, carbonic anhydrase; CAH3, chloroplastic CA identified from *C. reinhardtii*; CCM, carbon-concentrating mechanism; C_i, inorganic carbon; TAP, Tris acetate phosphate; TP, TAP medium minus acetate.

tant cells with CCM defects (e.g. *ca*-1). In bacterial and yeast systems such gene isolations can be routinely accomplished through techniques involving genomic complementation (Vollmer and Yanovsky, 1986; Rose, 1987; Fukuzawa et al., 1992). The recent availability of techniques for achieving relatively high transformation efficiencies in *C. reinhardtii* (Kindle, 1990) have allowed the potential for isolating targeted genes through genomic complementation using either random cosmid libraries (Purton and Rochaix, 1994) or an indexed cosmid library of *C. reinhardtii* DNA (Zhang et al., 1994).

In this study we demonstrate the use of the indexed cosmid library of Zhang et al. (1994) and genomic complementation to identify specific cosmid clones bearing a wild-type gene capable of restoring photosynthetic competence to mutant *ca*-1 under atmospheric CO_2 levels. We present DNA-sequencing data showing that the complementing gene encodes a CA apparently identical with the chloroplastic isoform of CA recently isolated and characterized by Karlsson et al. (1995). We conclude that the chloroplastic CA is essential to the operation of the CCM and, thus, photosynthesis in *C. reinhardtii*, at atmospheric levels of CO_2.

MATERIALS AND METHODS

Strains

Chlamydomonas reinhardtii strain *ca*-1–12–1C mt+ containing the nuclear *ca*-1–12–1C mutation was obtained from Dr. Robert Spreitzer (University of Nebraska-Lincoln). The strain was maintained on TAP medium (Gorman and Levine, 1965; Harris, 1989) in the dark because its original description reported it to be light-sensitive (Spalding et al., 1983a). Individual transformation experiments were carried out with cultures descended from isolated clones of mutant *ca*-1.

Indexed Library

The indexed cosmid library of *C. reinhardtii* DNA (containing 11,280 clones in the individual wells of 120 microtiter plates) and general approaches in using the library for genomic complementation have been described (Zhang et al., 1994).

Isolation of Pooled DNA from the Cosmid Library

For each microtiter plate in the library, a 96-well Bio-Block (Rainin, Woburn, MA) was inoculated with a drop of culture from each well on the plate using a 96-prong device that matched the configuration of the plate and the Bio-Block. Each Bio-Block well was filled with 1.8 mL of Luria broth medium supplemented with ampicillin (100 μg/mL). The 96-prong device was immersed in 70% (v/v) ethanol, flamed, and allowed to cool for approximately 2 min before inoculation. Inoculated Bio-Blocks were placed in a 37°C incubator for 16 h without agitation. Cultures from a single Bio-Block were transferred to a 1-L sterile flask and placed in a 37°C shaker at 250 rpm for an additional 3 h.

Cosmid DNA was isolated using a modified alkaline lysis procedure followed by cetyltrimethylammonium bromide precipitation (Ausubel et al., 1990). The resultant DNA was resuspended in buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and the yield of DNA was measured spectrophotometrically (model U-2000, Hitachi, San Jose, CA).

For the isolation of pooled cosmid DNA from individual rows and files of a "positive" microtiter plate (i.e. a plate containing a putative complementing gene), 12 Bio-Blocks were inoculated from the master plate. At the end of the period of stationary growth, media from one specific row or file of each of six Bio-Blocks were combined in a 1-L flask. The flask was shaken for 3 h at 37°C at 250 rpm to allow additional cell growth prior to DNA isolation.

Preparation of Autolysin

Autolysin was prepared using plate gametes of strains NO^- and $R3^+$ according to a procedure kindly supplied by Lisa Ellis (Washington University, St. Louis, MO). The two strains were spread onto TAP plates for production of dense lawns. After 5 to 7 d of growth under light, the plates were flooded with 5 to 10 mL of high-salt medium lacking a nitrogen source (Harris, 1989) and returned to the light bench for at least 1 h. The lawns were further loosened by scraping the plates with a bent, sterile glass rod, and the contents of one plate were poured onto a plate of the opposite mating type. Cells were allowed to mate in the light for 20 to 30 min. The mixture was poured into a 40-mL tube and centrifuged at 10,000*g* for 10 min in a rotor (JS13.1, Beckman). The supernatant containing autolysin was filtered through a 0.45-μm filter.

Transformation

Cells were grown to a density of 1 to 2 $\times$ 10^6 mL^{-1} in the dark in TAP medium supplemented with 10 mM Mops, pH 6.8. The cells were pelleted at 4000 rpm for 5 min in a rotor (JLA 10.5, Beckman) and resuspended in 1/50 to 1/100 the original culture volume of autolysin to dissolve cell walls. When the autolysin treatment was complete, as determined by microscopic examination of cells treated with 0.5% (v/v) Triton X-100, protoplasts were pelleted and resuspended to a concentration of 1 $\times$ 10^8 mL^{-1} in TAP plus 5% (w/v) PEG 8000. Then, 400 μL of cells was transformed with 10 μg of pooled cosmid DNA from a single microtiter plate of the indexed library. Transformation was accomplished using the glass bead vortexing method of Kindle (1990).

Given the complexity of the pooled plate DNA and the relatively low rate of transformation of *C. reinhardtii* (approximately 1 in 10^5 cells), perhaps as few as 5 to 10 of the 4 $\times$ 10^7 cells in a single transformation would be expected to be productively transformed with a copy of the targeted gene. Thus, a recovery period was implemented to ensure that no positive plates were missed because of the loss of rare transformants. Following transformation, the cells were transferred to TAP medium and incubated with gentle shaking in the dark for 2 d. Cells were then rinsed once with minimal medium (TP) and resuspended in 50 mL of

TP. Cells were then shaken in bright light for 7 d before being pelleted, rinsed once with TP, and spread over two to four plates containing TP. After 5 to 7 d of incubation under white light at 100 μmol s^{-1} m^{-2}, transformants were clearly visible. Subsequent transformations with pooled DNA isolated from each individual row and file of a positive microtiter plate allowed the single cosmid clone carrying the targeted gene to be pinpointed within the plate.

When an individual positive cosmid capable of complementing the mutant had been identified (see flowchart in Fig. 1), the region within the cosmid containing the targeted gene was defined more closely by digesting the cosmid with a number of restriction endonucleases and testing which ones abolished the ability of the cosmid to complement the mutant. Based on these results, small subclones of the cosmid containing the intact gene were obtained. By digesting these with *Eco*RI and *Sal*I, which were shown to inactivate the gene and to cut only one time within the complementing genomic fragment, small clones were obtained that had vector/insert junctions immediately adjacent to sequences within the gene. These were submitted for sequencing to the DNA Sequencing Core Facility in the Center for Biotechnology at the University of Nebraska-Lincoln. DNA sequence analyses and database searches were performed using the Genetics Computer Group software package (Devereux et al., 1984).

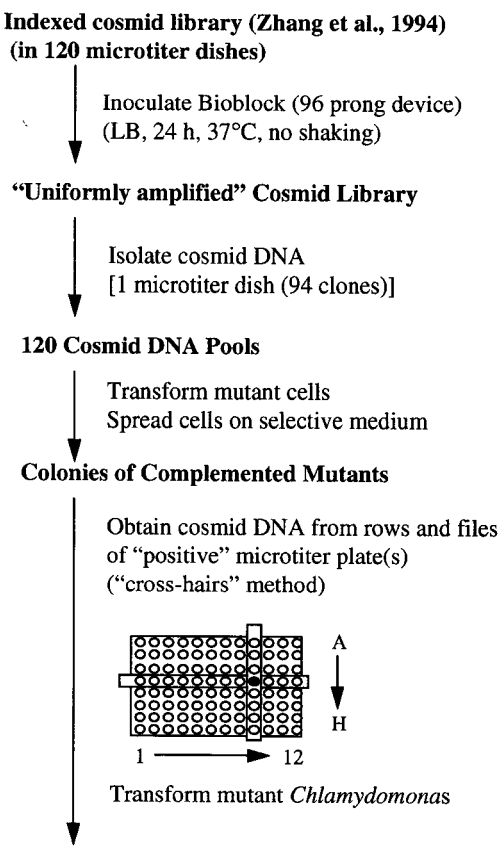

Figure 1. Use of the indexed cosmid library. The experimental steps involved in identifying a specific cosmid capable of complementing a mutant *C. reinhardtii* strain are summarized schematically.

DNA Gel-Blot Analyses

For gel-blotting experiments, DNA was fragmented with selected restriction endonucleases (GIBCO-BRL), size-fractionated on a 1% (w/v) agarose gel in 0.5 × Tris-borate-EDTA, and blotted onto a nylon filter (Boehringer Mannheim) using standard capillary transfer procedures (Sambrook et al., 1989). Membrane hybridizations were performed using a chemiluminescent detection system (Genius, Boehringer Mannheim).

Cloning and Sequencing of the Mutant *CAH3* Gene

The mutant gene was amplified from genomic DNA of strain *ca*-1 using high-fidelity *Pfu* DNA polymerase (Stratagene). Two pairs of primers were designed based on the sequence of the wild-type gene. One pair was used to amplify a fragment extending from position −522 to the *Eco*RI site in the middle of the third exon. A second pair was used to amplify a fragment extending from this *Eco*RI site to 450 bases past the end of the coding sequence. The amplified fragments were cloned. Three independent clones were sequenced to verify any differences observed in the fragments relative to the sequence of the wild-type gene.

Construction of Chimeric *CAH3* Genes

To locate and characterize the lesion in the mutant gene, constructs containing different combinations of mutant and wild-type gene fragments upstream and downstream of the *Eco*RI site in the third exon were used to transform strain *ca*-1. DNA (1 μg) from wild-type/mutant and mutant/wild-type chimeras was used to transform *ca*-1 and, thus, determine which part of the gene contained the mutation responsible for the high-CO_2-requiring phenotype of the mutant strain. A mock chimera was created by ligating the cloned 5′- and 3′-halves of the wild-type gene together. DNA from plasmid pCA-com, which contains the intact wild-type *CAH3* gene, was used as another positive control.

RESULTS

C. reinhardtii strain *ca*-1–12–1c mt+ containing the *ca*-1 mutation was transformed with DNA pooled from 94 clones in single microtiter plates of an indexed cosmid library of wild-type *C. reinhardtii* DNA (Zhang et al., 1994) using the glass bead transformation procedure (Kindle, 1990). Ninety of the 120 plates in the library were tested.

Two plates in the library (nos. 41 and 56) were identified that contained a clone capable of complementing the mutant (Table I). The "cross-hairs" procedure (Zhang et al., 1994) was used to identify the single row and the single file of plates 41 and 56 that contained the complementing gene (Fig. 1). This procedure pinpointed the single well in each

Table I. *Genomic complementation of the ca-1 mutant of C. reinhardtii*

Complementation results using pooled DNA from the *C. reinhardtii* indexed cosmid library.

Microtiter Plate No.	Total Transformants[a]
1–40	0
41	116
42–55	0
56	700[b]
57–90	0

[a] Ten micrograms of pooled DNA from one microtiter plate with 94 cosmids was used in each transformation. [b] Approximate value.

of these plates that contained the cosmid with the targeted gene (Table II). A comparison of the *Eco*RI and *Sma*I restriction patterns of the two independent cosmids from plate 41 (c41-D9) and 56 (c56-E7) demonstrated that they encompassed virtually the same piece of genomic DNA (Fig. 2). The insert in cosmid c56-E7 was slightly larger than the insert in cosmid c41-D9.

By determining which of several restriction enzymes did or did not abolish the ability of the cosmid to complement the mutant, we identified a 3-kb fragment that contained the intact targeted gene. DNA containing the intact gene was cloned into a Bluescript (Stratagene, LaJolla, CA) vector and was designated as pCA1-com. The genomic fragment contained in pCA1-com was subcloned into several overlapping clones of 300 to 500 bp in length. Both strands of the resulting clones were sequenced to obtain complete DNA sequence data (Fig. 3).

A FASTA search of GenBank (Devereux et al., 1984) using the complete, assembled sequence showed that the complementing genomic fragment encoded amino acid sequences with homology to several α-type CAs. Most importantly, the DNA fragment contained sequences corresponding almost exactly to the entire open reading frame of a cDNA clone encoding a chloroplastic CA that was

Table II. *Genomic complementation of the ca-1 mutant of C. reinhardtii*

Complementation results using cosmid DNA from rows and files (see Fig. 1).

Microtiter Plate No.	Row	File	Total Transformants[a]
41	1–8, 10–12		0
	9		TNTC[b]
41		A–C, E–H	0
		D	TNTC[b]
56	1–6, 9–12		0
	7		2800[c]
	8		15[d]
56		A–D, F–H	0
		E	1600[c]

[a] Ten micrograms of DNA was used in each transformation. [b] Too numerous to count. [c] Approximate value. [d] The small number of transformants may have been caused by contamination of an adjacent well by the positive cosmid during Bio-Block amplification of the cosmid clones.

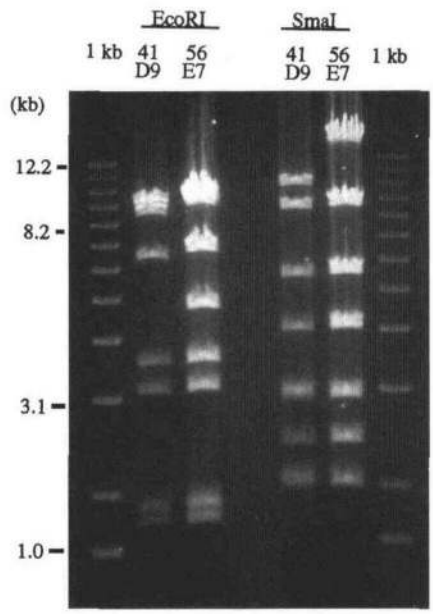

Figure 2. Two cosmids in the library capable of complementing mutant *ca*-1 encompass virtually the same segment of genomic DNA. DNA from cosmid D9 in plate 41 of the indexed library and cosmid E7 in plate 56 was digested with *Eco*RI and *Sma*I, and the restriction patterns were compared by pulse-field gel electrophoresis. Parameters: 120° angle, 14°C, 1% (w/v) SeaKem GTG agarose (FMC, Rockland, ME), 0.5-s switch time, 10-h run time, and 1× Tris-acetate-EDTA buffer. One-kilobar ladder markers (GIBCO-BRL) were used to flank the digests.

recently purified from *C. reinhardtii* (CAH3; Karlsson et al., 1995 [accession no. U40871]).

Comparison of the deduced translation product derived from the coding region of the wild-type *CAH3* gene isolated from our cosmid library with the protein encoded by the cDNA sequence deposited by J. Karlsson et al. (1995) revealed a single amino acid difference (His to Asn at position 189). Comparison with the extracellular CA polypeptides of *C. reinhardtii* (Fukuzawa et al., 1992) revealed that the same Zn^{2+}-liganded His residues and amino acids predicted to interact with Zn^{2+}-bound substrate molecules were conserved in the chloroplastic CA (Fig. 3).

To produce probes for hybridization analyses of genomic sequences with homology to the *CAH3* gene, the cloned 3-kb *Sma*I fragment was cut into two parts using restriction endonuclease *Eco*RI, and the two resulting segments were subcloned. The two halves of the fragment were labeled in vitro and hybridized separately to membranes carrying size-fractionated genomic DNA from wild-type strain 2137+ and the mutant strain *ca*-1 digested with selected restriction enzymes. Results presented in Figure 4, A and B, as expected, showed that the probes specific to the two halves of the *CAH3* gene detected a single 3-kb *Sma*I fragment in genomic DNA from wild-type cells. No differences were observed in the sizes of the restriction fragments of DNA from wild-type or mutant strains when hybridized with either probe. When the DNA was digested with *Eco*RI, probes specific to the two respective halves detected

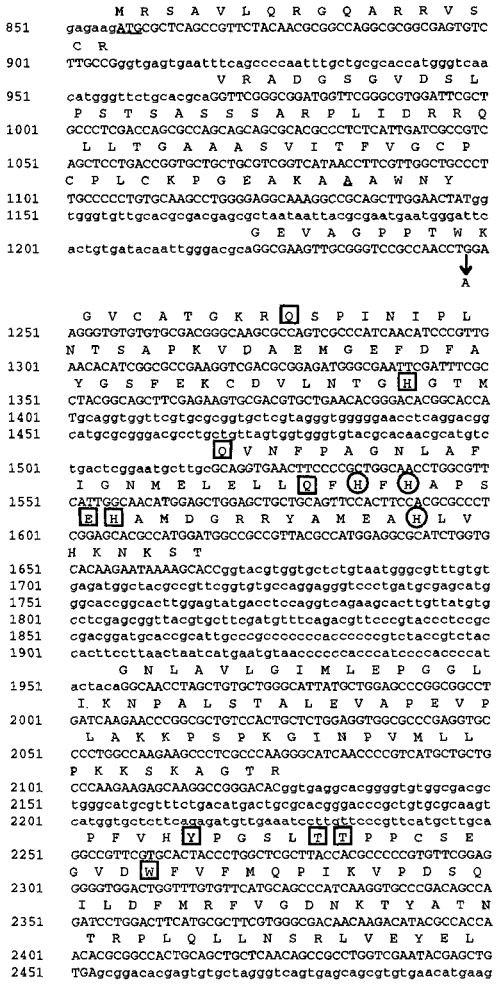

Figure 3. Coding sequence of the genomic clone that complements mutant *ca*-1. The ATG start codon is underlined. Additional sequence upstream and downstream of the coding sequence is available in GenBank (accession no. U73856). The translation of the sequence is shown above the nucleotide sequence. Introns are shown in lowercase. Intron-exon boundaries and the position of the first amino acid of the mature polypeptide (underlined) were deduced from the cDNA sequence of the protein purified by Karlsson et al. (1995) (accession no. U40871). The position of the G-to-A substitution that is responsible for the CA-deficient, high-CO_2-requiring phenotype of mutant *ca*-1 is indicated in the third exon by an arrow. Putative Zn^{2+}-liganded His residues that are conserved in both periplasmic isozymes of *C. reinhardtii* CA are circled, and residues that presumably participate in the hydrogen-bond network to bound solvent molecules are enclosed in squares (Fukuzawa et al., 1992).

different-sized restriction fragments in both strains (Fig. 4, A and B).

Comparison of the genomic and cDNA sequences suggested that the 933-bp coding region of the *CAH3* gene is interrupted by five introns varying in length from 24 to 74 bp and contains six exons varying in length from 50 to 203 bp. This information is summarized in Figure 4C, in which the pattern of intron and exon sequences in the *CAH3* gene is presented.

To test whether the *CAH3* gene in mutant *ca*-1 was the locus for the defect that caused the observed mutant phenotype in *ca*-1, and to determine which portion of the gene might contain the putative defect, we constructed chimeric *CAH3* genes that contained various combinations of the upstream and downstream halves of wild-type and mutant genes. These chimeric genes with upstream and downstream fragments joined at the *Eco*RI site in exon 3 (Fig. 4C) were then used to transform the *ca*-1 mutant. Transformations with the intact wild-type *CAH3* gene and DNA from the wild-type/wild-type and wild-type/mutant constructs produced about 700 colonies per plate. No transformants were obtained from the mutant/wild-type chimera, suggesting that the defect that causes the mutant phenotype of *ca*-1 was located in the 5′ portion of the *CAH3* gene.

Sequencing of the 5′ portion of the gene from the mutant strain revealed two differences relative to the wild-type gene sequence. One was a C-to-T transition at the 14th base in the first intron. Intron-differential reverse-transcriptionase PCR (Hongjun et al., 1990) was used to show that this mutation did not affect splicing of this intron in the *ca*-1 mutant (data not shown). The second difference was a G-to-A transition at the 26th base in the third exon in the second position of the TRP-86 codon. This mutation creates a termination codon (UAG) that results in the presumptive production of a polypeptide truncated after the 85th amino acid, just 13 amino acids beyond the processing site for the protein's transit peptide. This shortened product would not be expected to have enzymatic activity.

DISCUSSION

One approach to the molecular analysis of the CCM in *C. reinhardtii* has been to attempt to purify proteins that are candidates for having an important role in the CCM. Principal among these is the enzyme CA. More than one CA has been proposed to play a role in assisting the diffusion of C_i from outside the cell to the site of CO_2 fixation. Genes encoding the two extracellular CAs of *C. reinhardtii* have been isolated (Fukuzawa et al., 1990; Rawat and Moroney, 1991; Tachiki et al., 1992). The function of external CA may be to keep the external CO_2/HCO_3^- system in equilibrium and thus provide a continuous supply of CO_2 to the cell (Fett and Coleman, 1994). However, although extracellular CA activity increases sharply on transfer of wild-type *C. reinhardtii* from high CO_2 to atmospheric CO_2 levels, wall-less protoplasts that are ostensibly free of extracellular CA activity do not experience a dramatic reduction in their ability to accumulate internal C_i (Sültemeyer et al., 1990). Likewise, studies with membrane-impermeable CA inhibitors (Williams and Turpin, 1987) indicate that external CA is not necessary for efficient C_i uptake and utilization, even at high external pH, where HCO_3^- is the predominant C_i species. Thus, primary interest regarding CAs that might be involved in the CCM in *C. reinhardtii* has focused on internal, chloroplast-associated CAs. In spite of abundant evidence for their existence and importance in C_i assimilation (Husic et al., 1989; Husic and Marcus, 1994), these enzymes and the genes encoding them have remained elusive.

Our approach to the discovery of components essential to the operation of the CCM in *C. reinhardtii* has been to isolate

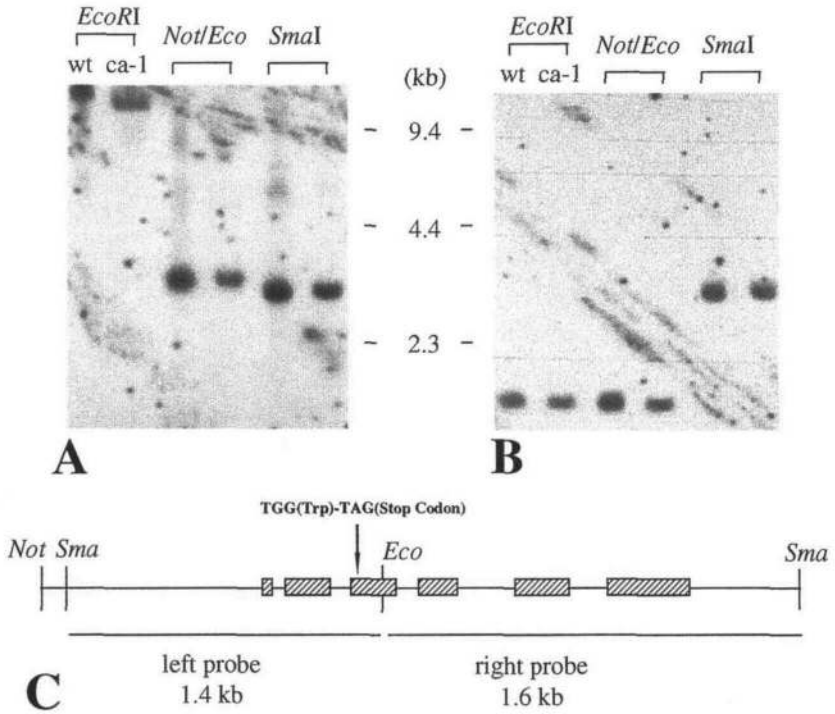

Figure 4. Southern analysis of the *CAH3* genes from wild-type and mutant *ca*-1 cells. A, Southern analysis of wild-type and mutant DNA digested with *Eco*RI and *Sma*I and probed with a cloned DNA fragment specific to the 5′ portion of the cloned *CAH3* gene (diagram in C). B, An identical blot probed with a cloned segment of the 3′ portion of the gene. C, Schematic representation of DNA restriction sites and the arrangement of exons (boxed areas) and introns of the *C. reinhardtii CAH3* gene.

genes capable of complementing defective genes in CCM-deficient mutants. We first targeted the *ca-1* mutant of *C. reinhardtii*, which exhibits a high-CO_2-requiring phenotype and is deficient in internal CA activity (Spalding et al., 1983a). This phenotype is determined by a single recessive nuclear mutation (Moroney et al., 1986), which is a prerequisite for gene isolation via genomic complementation.

Genomic complementation of the *ca*-1 mutant as described in this report led to the isolation of a gene encoding a CA. The identification of a nonsense mutation early in the coding region of this CA gene and the ability of the cloned wild-type gene to restore mutant cells to normal photosynthetic growth confirms that the nonsense mutation in the CA gene is responsible for the high-CO_2-requiring phenotype of the mutant. From these observations we can conclude unequivocally that a wild-type copy of a specific CA gene is required for photosynthetic growth in *C. reihardtii* at ambient CO_2 levels, and that the CA encoded by the gene is an essential component of the C_i-concentrating mechanism in this eukaryotic green alga.

The CA encoded by the complementing gene is clearly the same as the CA that was recently purified by Karlsson et al. (1995). The cDNA encoding this enzyme has been cloned (accession no. U40871) and named *CAH3*. The cDNA sequence contains a putative transit peptide, and western analysis of subcellular fractions of wild-type cells probed with an antibody to this CA indicate that it is located in the chloroplast (J. Karlsson, personal communication).

The finding that the gene that complements the *ca*-1 mutation encodes a CA was perhaps not the expected or logical outcome to our genomic complementation studies. Contrary expectations stemmed in part from recent research by Sültemeyer et al. (1995), which compared CA species in wild-type *C. reinhardtii* and the mutant, *ca*-1. They reported that in the *ca*-1 mutant the soluble form of chloroplast CA was either absent or present in nondetectable levels, whereas low but clearly measurable levels of CA activity could be found in the insoluble fraction of chloroplast lysates.

The apparent simultaneous but differential decrease in the levels of two distinct CA activities in chloroplasts of the *ca*-1 mutant might suggest that soluble and insoluble chloroplastic CAs are encoded by two separate genes and, furthermore, that these genes are differentially regulated by the product of the gene that is defective in the *ca*-1 mutant. Thus, a question arises as to how a lesion in a gene that encodes an insoluble chloroplast CA gene can be responsible for both a decrease in insoluble CA activity and the disappearance of soluble CA activity from the chloroplasts of mutant cells.

The simplest explanation to reconcile these findings is that the distinction between soluble and insoluble chloroplast CAs is a function of the methods used for subcellular fractionation. Karlsson et al. (1995) emphasized that extensive centrifugation is necessary to separate soluble and insoluble CA activities, since lighter membrane fragments and associated CA molecules can remain suspended in the supernatant fraction. They observed that salt treatment of the high-speed pellet caused some of the CA activity to be solubilized. If soluble chloroplast CA activity in fact represents solubilized or unsedimented *CAH3* CA, the lack of production of *CAH3* CA would account entirely for the disappearance of the soluble species of CA in the *ca*-1 mutant. It would also account for the apparent concomitant increase in soluble and insoluble chloroplast CA activities in wild-type cells on transfer to limiting CO_2 conditions.

Furthermore, it must be recognized that much may remain unknown with regard to the types of CAs present in *C. reinhardtii*. For example, the recent preliminary report of a large (approximately 142-kD) CA associated with the membrane fraction of *C. reinhardtii* chloroplasts (Carlson, 1995) may offer a logical explanation as to the residual CA activity found in the membrane fraction of extracts of mutant *ca*-1 and may point to the existence of yet one more gene that encodes a CA in this green alga.

Both eukaryotic green algae and prokaryotic cyanobacteria possess CCMs that are essential for efficient CO_2 fixation and photosynthesis at ambient CO_2 concentrations. Analyses of certain high-CO_2-requiring mutants and the use of CA inhibitors provided initial indications that CAs might be required for the function of the CCM (Badger and Price, 1994) in both eukaryotic and prokaryotic photosynthetic organisms living in aquatic environments. However, it was not until the isolation of the *icfA* gene from *Synechococcus* PCC7942 and its use to complement mutants with insertionally inactivated *icfA* genes that Fukuzawa et al. (1992) were able to provide definitive evidence that a CA was essential for CO_2 fixation in a prokaryotic cyanobacterium. In contrast to the α-type (i.e. animal-type) CA found in the present studies as an essential element of the (eukaryotic) *C. reinhardtii* CCM, the CA associated with the cyanobacterial CCM was distinctly of the β-type found in *Escherichia coli* (Sung and Fuchs, 1988; Guilloton et al., 1992) and the chloroplasts of higher plants (Fawcett et al., 1990; Roeske and Ogren, 1990). The significance of this observation is unknown but intriguing in light of the potential association of the cyanobacterial CA with the Rubisco-containing carboxysome structure (Fukuzawa et al., 1992; Badger and Price, 1994) and the present uncertainty of the number and subcellular locations of intracellular CAs in *C. reinhardtii*.

There is evidence that the pyrenoid body in the chloroplast stroma of green algae, including *C. reinhardtii*, contains both Rubisco and CA (Kuchitsu et al., 1991; McKay et al., 1991; Suss et al., 1995). This structure undergoes ultrastructural changes on transfer to low CO_2, and it has been proposed that the pyrenoid, like the carboxyosome of cyanobacteria, is the site of CO_2 fixation in these organisms (Kaplan et al., 1991; Badger and Price, 1994; Ramazanov et al., 1994; Sültemeyer et al., 1995). The *CAH3* gene product is clearly a candidate for a CA that might be associated with the pyrenoid. If this proves to be the case, the hypothesis that the pyrenoid plays an essential role in C_i accumulation would be strengthened.

Success in cloning the *CAH3* gene through genomic complementation of mutant *ca*-1 also opens the possibility of cloning genes that are defective in other CCM mutants of *C. reinhardtii*. The mutations that have been described to date fall into four complementation groups (Moroney et al., 1986, 1989). Results of genetic crosses involved in these studies indicated that mutant *ca*-1 shares the same complementation group with three other high-CO_2-requiring C_i mutants that were isolated independently using different mutagenesis procedures: *cia*-1, *cia*-2, and *cia*-3 (Moroney et al., 1986). Mutant *cia*-5 (Moroney et al., 1989) is a high-CO_2-requiring strain, which, because of its pleiotropic affect on a variety of CCM-associated functions, is surmised to potentially contain a defect in a master gene that controls multiple activities associated with the CCM. Mutant *cia*-4 (Moroney et al., 1986) occupies a separate complementation group from all other *C. reinhardtii* mutants that require high CO_2 for photosynthetic growth. The final complementation group also is represented by only one member, the *pmp*-1 mutant (Spalding, et al., 1983b). This mutant has provided strong evidence that a transporter for dissolved C_i (i.e. a C_i pump) is required for high levels of photosynthesis at low (atmospheric) concentrations of CO_2 in *C. reinhardtii*.

Characterization of the above-mentioned mutants has already provided valuable insight into the operation of the CCM. The increasing possibilities of isolating the genes underlying CCM defects in mutant cells through genomic complementation and other molecular and genetic approaches available in *C. reinhardtii* (Weeks, 1992) provides important new opportunities to advance understanding of the C_i-concentrating mechanism—a mechanism essential to life for the majority of photosynthetic microorganisms inhabiting the world's aquatic environments.

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