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Changes in gene expression during induction of the carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*

by

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INTRODUCTION

Photosynthesis and Photorespiration

Assimilation of inorganic carbon by plants, algae and cyanobacteria, occurs by a series of metabolic reactions known as the photosynthetic carbon reduction cycle, or PCRC, first described in 1957 (Bassham and Calvin 1957), and requires ATP and NADPH generated from light by membrane-associated photosystems. All organisms capable of photosynthesis utilize the PCRC to fix inorganic carbon, and in addition, some species of plants and algae have accessory pathways or systems that complement the PCRC making photosynthesis more efficient (described in detail later).

In the assimilatory reaction of the PCRC, ribulose 1,5-bisphosphate (RuBP) is carboxylated to generate two 3-carbon organic acids as the first stable products of photosynthesis. For every three CO_2 molecules fixed during steady-state photosynthesis, one 3-carbon acid is exported from the chloroplast for sucrose synthesis. The rest of the PCRC functions to regenerate RuBP. This pathway is known as C3 photosynthesis and the enzyme responsible for catalyzing net carbon assimilation is ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco).

In addition to CO_2, O_2 also serves as substrate for rubisco (Bowes et al. 1971, reviewed by Lorimer 1981) and both CO_2 and O_2 share the same catalytic site. Oxygen

inhibits photosynthesis directly because CO_2 and O_2 compete for the same catalytic site on rubisco, and indirectly because oxygenation of RuBP leads to a sequence of metabolic reactions known as the photosynthetic carbon oxidation cycle (PCOC) which results in net loss of already fixed carbon (reviewed by Ogren 1984, Tolbert 1981). RuBP oxygenation by rubisco and operation of the PCOC together are referred to as photorespiration, and the effect of photorespiration on RuBP carboxylation is demonstrated by experiments where photosynthetic carbon assimilation is measured as a function of O_2 concentration (Chollet and Ogren 1975, Forrester et al. 1966, Hesketh 1967, Ludwig and Canvin 1971). Most plants grown with air level CO_2 (0.03%) but with only 2% O_2, showed a marked increase in net CO_2 assimilation compared to plants grown with the same CO_2 but with air level of O_2 (21%). These results indicate that CO_2 availability is the limiting factor in photosynthesis and similarly point out the dilema concerning photosynthetic efficiency in an oxygen-containing atmosphere.

CO_2 Concentrating Systems

As stated earlier, certain plants and algae possess mechanisms to make photosynthesis more efficient. These natural systems increase photosynthetic efficiency by elevating the intracellular CO_2 concentration which results in decreased O_2-inhibition of photosynthesis and increased

net carbon assimilation under ambient CO_2 and O_2 concentrations. The very existence of systems that operate to suppress photorespiration demonstrates that the RuBP oxygenase activity of rubisco is dispensable to the plant. Similarly, findings that mutants lacking PCOC enzymes are able to grow normally as long as oxygenase activity is suppressed (Somerville and Ogren 1979, 1980, 1981) suggest the PCOC serves no purpose other than to metabolize the product of RuBP oxygenase activity, and is dispensable in the absence of oxygenase activity (Ogren 1984).

Because the substrates of rubisco are similar in structure and share the same catalytic site, and because oxygenase-deficient mutants have not been identified, it is generally accepted that oxygenase activity cannot be eliminated from rubisco without also eliminating carboxylase activity (Ogren 1984). For this reason, CO_2 concentrating systems have been the focus of many investigations as it appears this strategy is the only way to suppress photorespiration.

Three systems are known that increase the CO_2 concentration above that of air in the vicinity of rubisco: the C4 photosynthetic pathway (reviewed by Edwards and Huber 1981), Crassulacean acid metabolism (CAM) (reviewed by Osmond and Holtom 1981), and microalgal CO_2 concentrating mechanisms (reviewed by Badger 1987, Spalding 1989).

The C4 and CAM modes of photosynthesis

In C4 and CAM plants, the initial products of carbon fixation are four carbon acids produced by carboxylation of phosphoenolpyruvate (PEP) by PEP carboxylase. The C4 acids are used to supply CO_2 to rubisco, effectively increasing the CO_2:O_2 ratio, thereby suppressing the oxygenation of RuBP by rubisco.

In plants with C4 photosynthesis, carboxylation of PEP occurs in mesophyll cells and decarboxylation of the C4 acids and reassimilation of the CO_2 by the PCRC occurs in bundle sheath cells. In this way oxygenation of RuBP is suppressed because mesophyll cells lack most PCRC enzymes, most notably rubisco, and bundle sheath cells, which possess a complete PCRC, are supplied with an elevated CO_2 concentration. In some C4 species the CO_2:O_2 ratio is increased further as a result of diminished photosystem II O_2-evolution in the chloroplasts of bundle sheath cells. It can be seen that an elevated CO_2:O_2 ratio where the PCRC operates results from the separation of the two carbon assimilatory pathways and, in some species, from diminished photosynthetic O_2-evolution.

In contrast to C4 plants which exhibit physical separation of the C4 and C3 pathways, CAM plants utilize temporal separation of the C4 pathway from the PCRC to suppress photorespiration and conserve water. During darkness, when transpiration rates are low, stomates of CAM

plants open and CO_2 from the atmosphere is fixed by PEP carboxylase and stored as malic acid. In daylight, when ATP and NADPH are generated by the light reactions of photosynthesis and the PCRC enzymes are active, malate is decarboxylated to generate substrate for rubisco at concentrations sufficient to suppress oxygenase activity. Just as with C4 photosynthesis, CAM generates an elevated CO_2 concentration around rubisco thereby reducing photorespiration by suppression of RuBP oxygenase activity. Because the plant's stomates close during daylight, water loss is also limited.

The microalgal carbon dioxide concentrating mechanism

Microalgae possess only the C3 mode of photosynthesis but are able to concentrate CO_2 internally by the action of a relatively simple system involving perhaps only a few components. Like C4 and CAM photosynthesis, the microalgal CO_2 concentrating mechanism results in an elevated CO_2:O_2 ratio and suppressed RuBP oxygenase activity by rubisco.

Existence of a CO_2 concentrating mechanism in a microalga was first demonstrated in 1980 (Badger et al. 1980). Previously, studies (Berry et al. 1976) had shown that cells of the unicellular green alga *Chlamydomonas reinhardtii* exhibited photosynthetic characteristics that varied with the CO_2 concentration on which they were grown, with cells grown on low (air-level) CO_2 being better able to

utilize CO_2 for photosynthesis than cells grown on high (1 to 5%) CO_2. Low CO_2 cells exhibited high apparent affinity for CO_2 in photosynthesis, were insensitive to O_2-inhibition of photosynthesis, and were CO_2-saturated for photosynthesis by low external CO_2 concentrations. Photorespiration, as measured by glycolate synthesis, could only be measured when CO_2 was limiting for photosynthesis. High CO_2 cells exhibited photosynthetic characteristics typical of plants with C3 photosynthesis, including a relatively low apparent affinity for CO_2 and a high CO_2 compensation concentration. These characteristics suggested that different physiological states existed in low and high CO_2 cells and that "induction" occurred to allow the switch from C3 photosynthesis in high CO_2 cells to photosynthesis with C4-like characteristics in low CO_2 cells.

Although low CO_2 cells exhibited a greater affinity for CO_2 in photosynthesis than high CO_2 cells, rubisco from the two cell types did not demonstrate the same difference (Berry et al. 1976). Therefore, the photosynthetic characteristics observed for whole cells grown with either low or high CO_2 could not be attributed to changes in rubisco. The observed characteristics were, however, explained when Badger and coworkers demonstrated energy-dependent accumulation of inorganic carbon by low CO_2 *C. reinhardtii* cells. An elevated concentration of inorganic

carbon within low CO_2 cells, but not high CO_2 cells, was consistent with all of the previous measurements made for low versus high CO_2 cells in conditions of limiting external CO_2.

Components of the Microalgal CO_2 Concentrating Mechanism

Compared to the higher plant C4 and CAM pathways, the microalgal CO_2 concentrating systems appears to be relatively simple since it does not require an accessory pathway for carbon fixation nor does it require complex anatomical features or temporal regulation. It does, however, require ATP generated from photosynthesis (Spalding and Ogren 1982) and induction of at least two components: a saturable inorganic carbon transporter (Spalding and Ogren 1983, Spalding et al. 1983a) and internal carbonic anhydrase (Spalding et al. 1983b). Although there is overwhelming evidence for an inorganic carbon transporter, the transporter itself has not yet been identified. Also unclear is the intracellular location of the transporter; evidence exists for transport occuring at the plasma membrane (Sultemeyer et al. 1988, 1989) but active accumulation by isolated chloroplasts has also been measured (Moroney et al. 1987). Furthermore, controversy has existed as to what form of inorganic carbon (CO_2 or HCO_3^-) is substrate for transport, although recent evidence shows that low CO_2 cells selectively remove CO_2 from their culture

medium (Moroney et al. 1985, Sultemeyer et al. 1989). A role for internal carbonic anhydrase was established using a mutant deficient in this enzyme (Spalding et al. 1983b). The mutant was found to accumulate inorganic carbon to approximately 5 to 6 times that of wild type levels but was incapable of photoautotrophic growth on air level of CO_2, suggesting the internal inorganic carbon pool was unavailable for RuBP carboxylation. Dehydration of HCO_3^- to generate CO_2 is catalyzed by carbonic anhydrase and the mutant was in fact found to lack this activity, which explained how CO_2 could be limiting in the presence of a large pool of inorganic carbon.

In addition to an inorganic carbon transporter and internal carbonic anhydrase, evidence exists that two other components are required for operation of the microalgal CO_2 concentrating mechanism. First, induction of carbonic anhydrase activity external to the cell, either in the periplasmic space or the cell wall, has been shown to correspond with competency of cells to accumulate inorganic carbon (Bailly and Coleman 1988, Coleman and Grossman 1984, Coleman et al. 1984). This, together with the capacity of carbonic anhydrase to catalyze the reversible hydration and dehydration of inorganic carbon species, argues in favor of a requirement for this activity in inorganic carbon accumulation. That external carbonic anhydrase is a

component of the CO_2 concentrating mechanism is further supported by studies using specific inhibitors (Moroney et al. 1985). These studies indicate that external carbonic anhydrase is required for growth on low CO_2 since photosynthesis in these cells could be inhibited by blocking external carbonic anhydrase activity with the membrane impermeant CA inhibitor acetazolamide.

Evidence for another component is provided by a high CO_2-requiring mutant deficient in all of the changes observed in wild type cells switched from high to low CO_2 (Moroney et al. 1989). This mutant provides reason to believe that a regulatory component is required for induction of the microalgal CO_2 concentrating mechanism (Moroney et al.1989, Spalding et al. 1991). Since this mutant lacks all low CO_2-induced responses, it is unlikely that the mutation affects a functional component of the mechanism. It seems more likely that the mutation negatively affects a regulatory component that normally functions to signal the cell to switch physiological states. Although nothing is known about the signal responsible for induction, or how the algal cell senses the signal, it appears this mutant defines a genetic locus for a component of signal transduction, or for the signal itself.

Induction of the Microalgal CO_2 Concentrating Mechanism

Even though the microalgal CO_2 concentrating mechanism is probably simpler than higher plant CO_2 concentrating systems, much less is known about its components and operation than is known about the components and operation of the plant systems. Induction of the system, the events leading to the switch from C3 photosynthesis to C4-like photosynthesis, is poorly understood as well. For example, little is known about the changes in protein and gene expression that permit the switch from one physiological state to the other. This general lack of understanding is due largely to the fact that, thus far, only one gene coding for an inducible component of the CO_2 concentrating mechanism has been cloned. Identification of clones for the external carbonic anhydrase (Coleman and Bailly 1988, Fujiwara et al. 1990, this dissertation) has rapidly led to a better understanding of its expression and regulation (Bailly and Coleman 1988, Dionisi-Sese et al. 1990, Fukuzawa et al. 1990, this dissertation) which illustrates the utility of having molecular probes for the inducible components of the mechanism.

The present understanding of expression of the external carbonic anhydrase in inducing cells comes from having a molecular probe, but as already stated, this represents an exception, and a better indicator of the present

understanding of induction is demonstrated in studies where CO_2-regulated changes in protein expression have been studied. For example, Coleman and Grossman (1983) reported that subunit polypeptides of rubisco were synthesized at reduced rates during induction, and although others (Coleman and Bailly 1988, Spalding and Jeffrey 1989) have also observed the correlation between rubisco subunit synthesis and CO_2 concentration, the level of regulation remains unresolved. In other reports (Spalding and Jeffrey 1989, Geraghty et al. 1990, Manuel and Moroney 1988), numerous limiting CO_2-induced or up-regulated polypeptides have been identified in membrane and soluble cell fractions but, thus far, none of the polypeptides (other than periplasmic carbonic anhydrase) have been identified or proven to function in the CO_2 concentrating mechanism.

These examples point to the problem that identification of putative components of the mechanism far out-paces the assignment of functions to these components. Because the CO_2 concentrating mechanism is inducible and *C. reinhardtii* cells are easily labeled *in vivo*, identification of potential components is made relatively easy. For this reason, there are more putative components of the CO_2 concentrating mechanism than proven ones. Nevertheless, the inducible nature of the microalgal system should facilitate the eventual identification and characterization of all its

components.

The aim of present and future research in the microalgal CO_2 concentrating mechanism is to understand at the molecular level its components and regulation. By studying responses at the molecular level in inducing cells we should eventually be able to identify all of the components and activities necessary for operation of the microalgal CO_2 concentrating mechanism. After the components and requirements of active CO_2 accumulation by microalgae are fully understood, the possibility of transferring the system to crop plants with C3 photosynthesis can be addressed. The beneficial effect that raising intracellular CO_2 concentrations has to the efficiency of photosynthesis, together with the relative simplicity of the microalgal CO_2 concentrating system, make this a worthwhile and important system to study. It is hoped that the findings reported in this dissertation contribute to the present understanding of events during induction of the CO_2 concentrating mechanism.

Explanation of Dissertation Format

In this dissertation, I have studied changes in protein and gene expression during induction of the CO_2 concentrating mechanism in wild type and mutants of *C. reinhardtii*. Specifically, I investigated regulation of synthesis of the rubisco subunits (Part I) and of the

external carbonic anhydrase in a cell wall-deficient mutant (Part III) and in a mutant unable to adapt to low CO_2 (Part IV). Isolation of a cDNA for the low CO_2-induced external carbonic anhydrase (Part II) provided a probe to study carbonic anhydrase gene expression so I could determine where in its pathway of expression regulation acted.

This dissertation is presented in the alternate format consisting of four parts and a brief summary. Literature citations used in the introduction to the dissertation are listed following the summary. All of the work reported in Parts I and III has been submitted for publication and a substantial amount of the work reported in Part IV has already been published. Thomas L. Winder was the principal investigator and Dr. Martin H. Spalding served as the major Professor.

PART I. TRANSLATIONAL REGULATION OF THE LARGE AND SMALL SUBUNITS OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE DURING INDUCTION OF THE CO_2 CONCENTRATING MECHANISM IN *CHLAMYDOMONAS REINHARDTII*

ABSTRACT

In conditions of limiting external inorganic carbon, the unicellular alga *Chlamydomonas reinhardtii* induces a mechanism to actively transport and accumulate inorganic carbon within the cell. A high internal inorganic carbon concentration enables the cell to photosynthesize efficiently with little oxygen inhibition, even in conditions of limiting external inorganic carbon. A correlation between limiting inorganic carbon-induced induction of the CO_2 concentrating mechanism and decreased synthesis of the large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase has been observed. Cells that had been transferred from elevated CO_2 to limiting CO_2 exhibit transient declines of label incorporation into both subunit polypeptides. The decrease in synthesis of both large and small subunits is shown to be a result of specific and coordinated down-regulation of translation of both subunits.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; rubisco) catalyzes the first steps of the photosynthetic carbon reduction cycle or the photorespiratory pathway when it reacts with CO_2 or O_2, respectively. Rubisco consists of eight catalytic large subunits (L) and eight small subunits (S) of undetermined function. Precursor S subunits are encoded by a multigene family (*rbc*S) on the nuclear genome and are post-translationally transported to the chloroplast where they are processed to mature form by proteolytic removal of an amino terminal transit peptide (reviewed by Schmidt and Mishkind 1986). L subunits are encoded by the single copy *rbc*L gene of the chloroplast genome and are translated by plastid ribosomes. Assembly of the rubisco holoenzyme complex occurs in the chloroplast and is aided by rubisco subunit binding protein (reviewed by Roy 1989). Because pools of unassembled subunits do not accumulate in the chloroplast, successful assembly of holoenzyme requires that synthesis of subunits be regulated to ensure that each is present at stochiometric levels.

Coordinated accumulation of rubisco subunits may be achieved by regulatory controls at transcription (reviewed by Tobin and Silverthorne 1985), post-transcriptional mRNA stability (Fritz et al. 1991), translation (e.g. Berry et

al. 1988), and post-translational turn-over of unassembled polypeptides (e.g. Schmidt and Mishkind 1983). Within a species, control over synthesis of either subunit may be exerted at more than one level in a heirarchial manner (Berry et al. 1986, Winter and Feierabend 1990), and between species entirely different regulatory controls may act. Control mechanisms used by plants and algae to cause coordinated accumulation of rubisco subunits in the event of shifts in illumination (Berry et al. 1988, Berry et al. 1986, and reviewed by Tobin and Silverthorne 1985), developmental programs (Deng and Gruissem 1987, Mullet and Klein 1987, Nikolau and Klessig 1987), nonsense mutations of the *rbc*L gene (Hildebrant et al. 1984, Spreitzer et al. 1985) or in the event of interference with chloroplast or cytoplasmic protein synthesis (Radetzky and Zetsche 1987,Schmidt and Mishkind 1983, Winter and Feierabend 1990) have been elucidated.

Because changes in illumination have been shown to result in specific regulation of rubisco biosynthesis, it should not be surprising that other environmental factors should have similar effects. One environmental factor of central importance to the activity of rubisco is CO_2 concentration, and one well studied system to cope with limiting CO_2 is the CO_2 concentrating mechanism of algae. *Chlamydomonas reinhardtii* and other algae possess an

inducible system that functions to raise intracellular C_i concentrations in conditions of low external C_i (reviewed in Badger 1987). High internal C_i concentrations effectively increase the supply of substrate CO_2 for rubisco and diminish rubisco oxygenase activity thereby reducing photorespiration. The system is absent from cells cultured under elevated CO_2 (1-5% CO_2 in air) but is fully operational within 8 hrs in limiting external CO_2 (Coleman et al. 1984, Spalding and Jeffrey 1989). Capacity for active C_i transport (Spalding et al. 1983, Sultemeyer et al. 1989) and induction of carbonic anhydrase (Bailly and Coleman 1988) coincide with increased affinity for C_i in photosynthesis.

C. reinhardtii cells adapting to limiting CO_2 induce synthesis of carbonic anhydrase and several other polypeptides (Spalding and Jeffrey 1989), and interestingly, specifically depress synthesis and/or accumulation of rubisco L and S (Coleman and Grossman 1983, and this study). Similar declines in synthesis of L and S polypeptides coincidental with establishment of the CO_2 concentrating mechanism suggest *C. reinhardtii* cells somehow down-regulate biosynthesis of rubisco subunits in a coordinated manner in response to limiting CO_2 concentrations. This observation has been investigated further and it has been determined that these cells control accumulation of both subunits by

specifically limiting their translation. This is in marked contrast to the post-translational control mechanism used in *C. reinhardtii* cells deficient in chloroplast protein synthesis (Schmidt and Mishkind 1983) or with *rbcL* nonsense mutations (Spreitzer et al. 1985), and demonstrates a second means by which *C. reinhardtii* cells can exert control over rubisco biosynthesis to achieve coordinated accumulation of its subunits.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii wild type strain 2137 (Spreitzer and Mets 1981) and cell wall-deficient mutant CW-15 (obtained from Dr. R. Togasaki, Indiana University) were cultured in minimal salts medium as previously reported (Geraghty et al. 1990). Cells were cultured on an orbital shaker under aeration with elevated CO_2 (5% CO_2 in air). Cultures to be switched from elevated CO_2 to limiting CO_2 (0.03% CO_2 in air) for induction experiments were centrifuged from the medium and resuspended in minimal salts medium buffered for low CO_2 aeration.

In Vivo Labeling Experiments

Cells cultured with elevated CO_2 or with limiting CO_2 for 4, 8 or 24 hrs were collected and washed with minimal salts medium lacking sulfate and resuspended in an original culture volume of minus-sulfate medium 4 hours before harvesting. Label (100 μCi [^{35}S]sulfuric acid) was added 30 min before harvesting. Cells were harvested and broken as previously described (Geraghty et al. 1990) and soluble proteins were separated from membranes by centrifugation (48,000xg, 20 min). For pulse-chase experiments, cells cultured with elevated CO_2 or with elevated CO_2 followed by 4 hours of limiting CO_2 were washed and resuspended in

minus-sulfur medium exactly as in the 30 min labeling experiments. The pulse-chase protocol developed by Schmidt and Mishkind (1983) was followed. Cells were given a 5 min pulse of 1 mCi/ml [^{35}S]sulfuric acid (carrier-free; 43 Ci/mg ^{35}S at 100% isotopic enrichment) followed by up to 60 min chase with 10 mM Na_2SO_4.

Protein Gels and Autoradiography

All protein samples were concentrated and unincorporated label eliminated from the samples using Amicon Centricon 10 concentrators. SDS polyacrylamide gels (10-18%) were run using equal amounts of radioactive protein per lane. Protein was transferred to nitrocellulose sheets (Geraghty et al. 1990) and autoradiographed.

RNA Analysis

Total RNA was prepared from cells cultured with elevated CO_2 or induced by limiting CO_2 concentrations for 1, 2, 4, 8 or 24 hours as previously described (Geraghty et al. 1990). RNA (10 µg/lane) was electrophoresed in formaldehyde-agarose gels and transferred to nitrocellulose sheets (Fourney et al. 1988). RNA gel blots were probed using standard methods. Rubisco small subunit mRNAs were detected with the CS2.1 *rbcS2* cDNA clone (kindly provided by Dr. M. GoldSchmidt-Clerèmont) which is 98.5% homologous over 90% of its length to the *rbcS1* sequence and therefore

hybridizes nearly equally as well to both mRNAs (Goldschmidt-Cleremont and Rahire 1986). Large subunit mRNA was detected with the R15.4 sub-fragment of the *C. reinhardtii* chloroplast R15 clone (kindly provided by Dr. R. Spreitzer). Quantitation of *rbc*S1 and *rbc*S2 mRNA abundance from Northern blots was performed by liquid scintillation spectroscopy of bands excized from the blot.

RESULTS AND DISCUSSION

Decreased Synthesis of Rubisco Subunits

The present study confirms an earlier observation (Coleman and Grossman 1983, Spalding and Jeffrey 1989) that, when *C. reinhardtii* cells adapt from a CO_2-enriched environment to a CO_2-limiting environment, synthesis of rubisco L and S temporarily decline but then return to the levels in high CO_2-grown cells. *C. reinhardtii* cells cultured with elevated CO_2 were adapted to limiting CO_2 for 4, 8 or 24 hours and *in vivo* labeled at each time point in order to monitor accumulation of newly synthesized polypeptides. Autoradiograms of SDS/PAGE-separated soluble protein from experiments using either wild type (2137) or a cell wall-deficient mutant (CW-15) are shown in Figure 1. Both *C. reinhardtii* strains had diminished synthesis and/or accumulation of L and S between 4 and 8 hours of limiting CO_2 growth, but between 8 and 24 hours, newly synthesized L and S again accumulated as in high CO_2-grown cells. Decreased L and S synthesis and/or accumulation in 4 and 8 hr cells as measured by diminished *in vivo* labeling could result from: 1) decreased abundance of translatable mRNA for one or both of the subunits, 2) inhibition of translation elongation or initiation, or 3) rapid degradation of one or both of the newly made subunits.

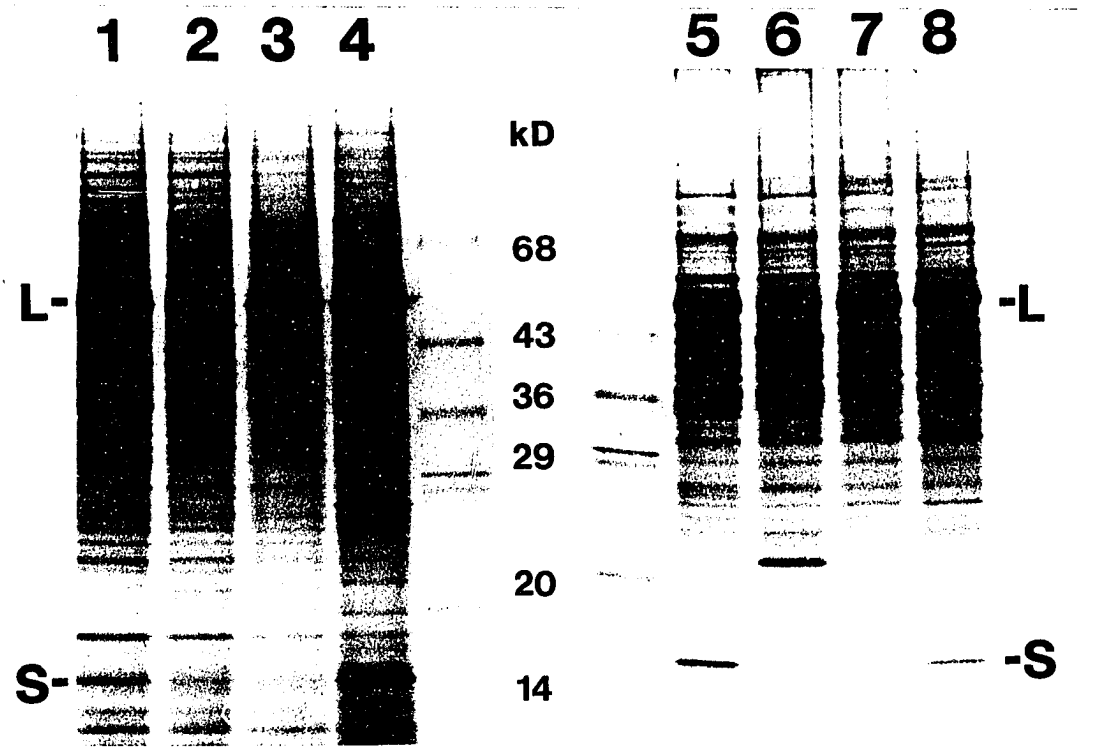

Figure 1. *In vivo* labeled polypeptides in the soluble fraction of *C. reinhardtii* strains CW-15 (lanes 1-4) and 2137 (lanes 5-8) cultured with elevated CO_2 (lanes 1 and 5) or with limiting CO_2 for 4 hr (lanes 2 and 6), 8 hr (lanes 3 and 7) and 24 hr (lanes 4 and 8). Polypeptides were labeled for 30 min prior to harvesting. Radioactive polypeptides were transferred to nitrocellulose and autoradiographed. Positions of rubisco large (L) and small (S) subunit polypeptides are indicated

Experiments have been conducted aimed at determining which stage of rubisco subunit biosynthesis is regulated to cause the depression in subunit labeling in limiting CO_2-adapting *C. reinhardtii* cells.

Measurement of Pool Sizes for Rubisco Subunit mRNAs

Adjustment of *rbc*L and *rbc*S mRNA pool sizes is the principal way plants and algae coordinate L and S protein levels during developmentally induced changes (reviewed by Tobin and Silverthorne 1985). To determine if *C. reinhardtii* cells undergoing adaptation to limiting CO_2 use this level of control to regulate rubisco levels, accumulation of hybridizable *rbc*S and *rbc*L mRNAs was monitored for strains 2137 and CW-15 adapted to limiting CO_2 for 1, 2, 4, 8 and 24 hours as well as for high CO_2-grown cells (Figure 2). Based on measurements of filter-bound radioactivity, the more abundant transcript (*rbc*S2) of the two-gene *rbc*S family remained essentially unchanged in both strains over the time-course; the only exception being the 2 and 4 hr time points in which small (20-30%) declines were found (Figure 2 lanes 3 and 4, respectively). A more significant change in abundance was observed for *rbc*S1 mRNA in CW-15 cells (but not in 2137 cells): *rbc*S1 mRNA in CW-15 cells at 2 hr, 4 hr, 8 hr and 24 hr was only 47%, 28%, 28% and 61% of the level found in high CO_2 grown cells, respectively.

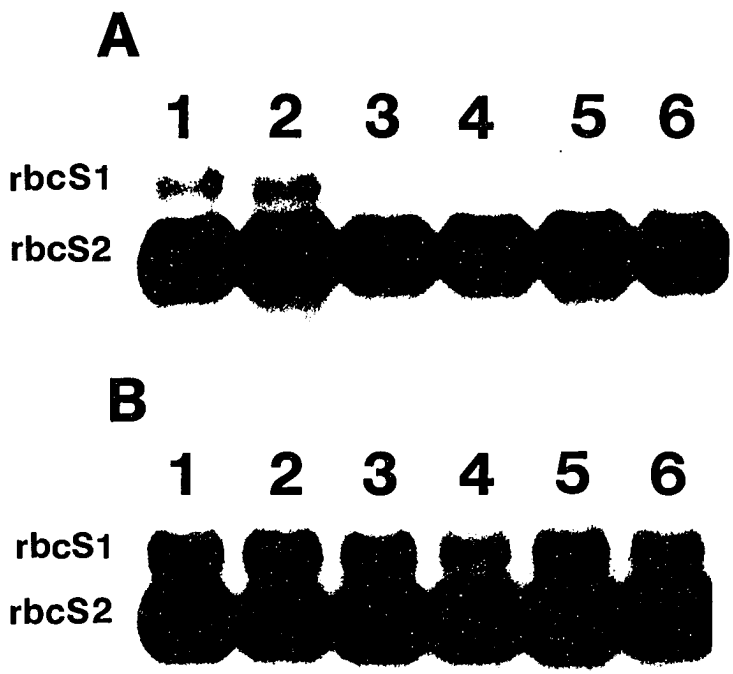

Figure 2. *RbcS* mRNA accumulation in *C. reinhardtii* strains CW-15 (panel A) and 2137 (panel B) cultured with elevated CO_2 (lane 1) or with limiting CO_2 for 1 hr (lane 2), 2 hr (lane 3), 4 hr (lane 4), 8 hr (lane 5) and 24 hr (lane 6). Cellular RNA was separated by electrophoresis in formaldehyde-agarose gels, transferred to nitrocellulose and probed with a cDNA for *rbcS2* which hybridizes to both mRNAs

Involvement of *rbc*S1 mRNA regulation is not suspected in control over S polypeptide levels in our study, since we found constitutive expression of *rbc*S1 mRNA in wild type cells and because *rbc*S2 mRNA in both strains remains essentially unchanged in abundance and contributes most of the mRNA to the *rbc*S mRNA pool. We conclude, therefore, that the decline of *rbc*S1 mRNA in CW-15 is a strain-specific effect: if it played a significant role in the down-regulation of S polypeptide synthesis, we would expect the decline to be seen in 2137 cells as well. Nevertheless, in veiw of evidence (Goldschmidt-Cleremont and Rahire 1986, Johanningmeier 1988) that *rbc*S1 under other conditions is regulated at the level of mRNA abundance, it remains intriguing that the patterns of *rbc*S1 mRNA abundance and S accumulation appeared to be exactly coordinated.

No evidence was seen for control of *rbc*L mRNA pool size in limiting CO_2-adapting *C. reinhardtii* cells since *rbc*L mRNA abundance did not vary during the time course of adaptation to limiting CO_2 (no data shown). Abundance of *rbc*L mRNA does not vary at any time of the cell cycle in synchronous *C. reinhardtii* cells (Matsuda and Surzycki 1980), suggesting regulation of L synthesis by mRNA abundance is not common in *C. reinhardtii*.

Pulse-Chase Protein Labeling Studies

Because accumulation of newly synthesized L and S in limiting CO_2-adapting *C. reinhardtii* cells was not correlated with their corresponding mRNAs levels, translational or post-translational controls must act in these cells to cause the observed decline in rubisco subunit synthesis. Depression of synthesis of L and S could be coordinated if one subunit was regulated translationally while the other was rapidly turned-over post-translationally. Alternatively, both subunit polypeptides could be synthesized at normal rates but rapidly degraded so that synthesis appears to be decreased. In higher plants, degradation of excess mature S polypeptide seems to allow for the most rapid equilibration of subunit concentrations (Winter and Feierabend 1990), and this strategy is utilized in algae when L polypeptide does not accumulate (Hildebrant et al. 1984, Radetzky and Zetsche 1987, Schmidt and Mishkind 1983, Spreitzer et al. 1985).

Pulse-chase experiments with either high-CO_2 grown or 4 hr limiting-CO_2 adapted cells would indicate if one (or both) of the subunit polypeptides was being synthesized but rapidly degraded. High CO_2-grown cells pulsed for 5 min synthesized L and S that were stable throughout the 60 min chase period (Figure 3; lanes 1-5), but when cells were cultured in limiting CO_2 for 4 hr prior to the pulse

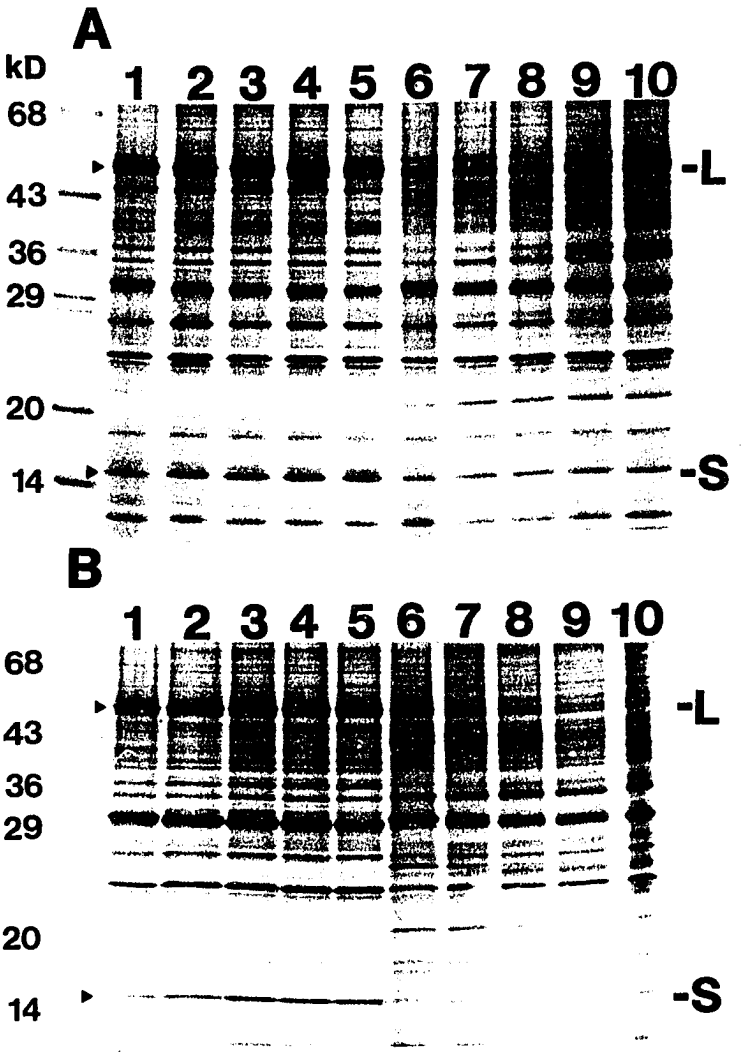

Figure 3. Pulse-chase labeled polypeptides found in the acetone insoluble fraction of *C. reinhardtii* strains CW-15 (panel A) and 2137 (panel B). Pulse-chase labelings of cells cultured with elevated CO_2 (lanes 1-5) and cells switched from elevated CO_2 to limiting CO_2 for 4 hr (lanes 6-10) are shown. Polypeptides from cells pulse-labeled for 5 min (lanes 1 and 6) and from cells chased for 10 min (lanes 2 and 7), 20 min (lanes 3 and 8), 30 min (lanes 4 and 9), and 60 min (lanes 5 and 10) were analyzed by SDS/PAGE and autoradiography. Positions of rubisco large (L) and small (S) subunit polypeptides are indicated with arrowheads in the molecular weight marker lane

(Fig 3; lanes 6-10), incorporation of radioactivity into L and S was diminished in comparison to high CO_2-grown cells and in comparison to other polypeptides made by these cells. Apparently, a small amount of L and S was synthesized during the 5 min pulse in 4 hr limiting CO_2-adapted cells, and these new polypeptides were stable throughout the 60 min chase period. Since synthesis of L and S is diminished in 30 min labeling periods (see Fig. 1), any contribution of turn-over should be seen in the 60 min chase. In fact, post-translational turnover of S in the absence of L synthesis has been demonstrated to begin 10 min after pulse labeling, and to be essentially complete after 60 min (Schmidt and Mishkind 1983, Spreitzer et al. 1985). The results presented in Figure 3 clearly indicate synthesis of S followed by rapid degradation in the absence of L is not occuring. Rather, it appears translation of both L and of S is diminished, and that the level of control for S is the same as that for L.

It is not known whether the translational control in these cells involves inhibition of elongation or initiation, or both as in the case of amaranth seedlings (Berry et al. 1988). However, rapid degradation of newly synthesized subunit polypeptides as well as control of mRNA levels have been ruled-out as mechanisms used to coordinately down-regulate synthesis of L and S during adaptation to limiting

external C_i.

When accumulation of L was blocked in algae, either by antibiotic treatment (Radetzky and Zetsche 1987, Schmidt and Mishkind 1983) or by genetic defects (Hildebrant et al. 1984, Schmidt and Mishkind 1983, Spreitzer et al. 1985), S synthesis proceeded but unassembled S was unstable, with a half-life of only 7.5 minutes (Schmidt and Mishkind 1983). This type of control over biosynthesis of rubisco has been regarded as a final adjustment mechanism to attain stochiometric levels of L and S (Winter and Feierabend 1990). Control by translational regulation is in marked contrast to this mechanism and appears to be like that found in higher plants shifted from light to dark (Berry et al. 1988). Translational regulation of L and S by limiting CO_2 adapting cells might be regarded as a coarse control, functioning in response to drastic changes in growth conditions that induce major shifts in metabolism of the cell. Therefore, whether stochiometric accumulation of L and S is attained by transcriptional, post-transcriptional, translational or post-translational mechanisms may be dependent not only upon genetic determinants but also upon the nature of the stimulus affecting the altered rubisco levels.

Induction of the CO_2 concentrating mechanism of *C. reinhardtii* involves changes in gene transcription (Bailly

and Coleman 1988), protein synthesis (Bailly and Coleman 1988, Coleman and Grossman 1983) and energy-requiring C_i transport (Sultemeyer et al. 1989). As a consequence of these demands for energy and components to build macromolecules placed upon the cell by this growth condition, production of expendable macromolecules might be temporarily interupted in a specific or a general manner. Although rubisco activity is not dispensable to the cells in our experimental conditions, the stability of the holoenzyme may make production of new subunits temporarily expendable, particularly if cell growth and/or division is arrested. Evidence suggests that cell division possibly is interupted for 4 to 6 hrs immediately after transfer of cells from elevated CO_2 to limiting CO_2 (L. Marek, unpublished observations).

Although it is only possible to speculate on the causes for diminished demand for new rubisco subunits in limiting CO_2-adapting cells, this study clearly shows diminished rubisco subunit synthesis results from a specific translational control that similarly affects both subunits.

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PART II. cDNA CLONING OF PERIPLASMIC CARBONIC ANHYDRASE FROM *CHLAMYDOMONAS REINHARDTII*

ABSTRACT

Chlamydomonas reinhardtii and other algae exhibit very different photosynthetic characteristics when grown with air-level of CO_2 than when grown with 1 to 5% CO_2. Low CO_2-grown cells exhibit high apparent affinity for CO_2 in photosynthesis, have a near zero CO_2 compensation concentration, lack O_2-inhibition of photosynthesis and have no measurable photorespiration. The ability of low CO_2-grown cells to efficiently utilize CO_2 in photosynthesis results from activity of CO_2 transport and extracellular carbonic anhydrase. This report describes the cloning of a cDNA for the CO_2-regulated extracellular carbonic anhydrase from low CO_2-grown *Chlamydomonas reinhardtii* cells.

INTRODUCTION

Chlamydomonas reinhardtii and other algae exhibit higher apparent affinity for inorganic carbon (C_i; CO_2 + HCO_3^-) when grown with air level of CO_2 than when grown with air enriched to 1-5% CO_2. Activity of carbonic anhydrase (CA) (Badger et al. 1980, Moroney et al. 1985) and operation of a CO_2 concentrating mechanism (reviewed by Badger 1987, Spalding 1989) in low CO_2-grown cells, but not in high CO_2-grown cells, result in CO_2 accumulation and very efficient utilization of CO_2 in photosynthesis. Efficient use of CO_2 in photosynthesis is paralleled by appearance of CA activity (Coleman and Grossman 1984), and is blocked by CA-specific inhibitors (Moroney et al. 1985), providing evidence that CA is at least partly responsible for increased cellular affinity for C_i in photosynthesis. Periplasmic CA (perCA), located external to the cell, either in the periplasmic space or associated with the cell wall, catalyzes the dehydration of HCO_3^- to replenish the CO_2 pool at alkaline pH. Rapid resupply of CO_2 is necessary to sustain high rates of photosynthesis because CO_2 (and not HCO_3^-) is preferentially utilized (Sultemeyer et al. 1989), and because the uncatalyzed equilibration between CO_2 and HCO_3^- is slow.

CA activity located intracellularly is also believed to be necessary for efficient use of C_i by low CO_2-grown cells

(Moroney et al. 1987a, Spalding and Portis 1985), and measurement of intracellular CA activity (Husic et al. 1989, Moroney et al. 1987b) and identification of polypeptides immunologically related to, but distinct from, the periplasmic CA (Husic et al. 1989) have been reported. Intracellular CA, however, represents only a small proportion of the total CA in low CO_2-grown cells, the majority being located at the cells external surface (Coleman et al. 1984, Kimpel et al. 1983, Moroney et al. 1985, Yagawa et al. 1986).

Nearly all of the cellular CA activity can be measured using intact cells, and when cell walls are removed (Yagawa et al. 1986) or when a cell wall-deficient mutant is used (Coleman et al. 1984, Kimpel et al. 1983), essentially all of the CA is released to the culture medium. The cell wall-deficient mutant CW-15 apparently compensates for its inability to retain CA protein in the periplasmic space by over-producing perCA mRNA. Compared to a wild type strain, CW-15 accumulates about two-fold more perCA transcript, protein and activity following exposure to limiting CO_2 for 24 hrs (Part III, this dissertation).

Over-accumulation of perCA transcript by the cell wall-deficient strain, as well as the accumulation of perCA transcripts exclusively in low CO_2-grown cells, were taken advantage of to clone a cDNA for this gene. It was reasoned

that a cDNA library derived from poly[A] RNA isolated from limiting CO_2-induced CW-15 cells would be enriched in copies of the perCA transcript and, therefore, should work well in a differential screening strategy to try and find the limiting CO_2-specific cDNA.

MATERIALS AND METHODS

Algal Cultures and Poly[A] RNA Isolation

Chlamydomonas reinhardtii cell wall-deficient mutant CW-15 (obtained from Dr. R. Togasaki, Indiana University) was cultured in liquid minimal salts medium (Geraghty et al. 1990) photoautotrophically on a gyratory shaker. Cultures were aerated with 5% CO_2 in air (high CO_2-grown) and were induced to accumulate perCA transcript by switching the CO_2 concentration to that of air (0.03%).

Following 4 hours of air-induction, cultures were harvested and total RNA was isolated as previously described (Geraghty et al. 1990). Poly[A] RNA for library construction and for probe synthesis was fractionated using poly[U] Sephadex affinity column chromatography (Lissemore et al. 1987). The "4 hr air-induced" cDNA library was made by CloneTech Laboratories, Inc., Palo Alto, CA using the lambda vector gt10.

cDNA Cloning

Probes for differential screening were synthesized with M-MLV reverse transcriptase (Bethesda Research Laboratory) following the suppliers instructions except that 100 μCi each of [^{32}P]dCTP and [^{32}P]dATP (3000 Ci/mMol) were included. Template for the reverse transcriptase reactions was 1 μg of poly[A] RNA from either 4 hr air-adapted cells

(+ probe) or cells cultured continuously in 5% CO_2 (- probe). Unincorporated radionucleotides were removed from the cDNA by chromatography through Bio-Gel P-60 (100-200 mesh) hydrated in 20 mM Tris-HCl (pH 7.5), 1 mM Na_2 EDTA, 0.1% SDS and 5 µg/ml yeast RNA. Hybridization of probes to duplicate plaque lifts of the cDNA library was performed using standard techniques (Sargent 1987). Autoradiograms of the + and - probed plaques were compared to one another and plaques that hybridized to + probe but not to - probe were rescreened at lower plaque density. cDNA inserts of the gt10 clones were subcloned into the phagemid pBluescript (Stratagene Inc., La Jolla, CA) for further analysis.

Putative air-specific cDNAs were radiolabeled and hybridized to Northern blots (Fourney et al. 1988) containing RNA from either 5% CO_2-grown cells or air-adapted cells to further verify their specificity to limiting CO_2 adapted cells. A partial genomic clone for *C. reinhardtii* perCA (Bailly and Coleman 1988; kindly provided by Dr. J. Coleman, University of Toronto) was radiolabeled and used to show that all our air-specific clones were perCA.

The perCA cDNA clone was sequenced using an Applied Biosystems Model 373A automated DNA sequencer by the Iowa State University Nucleic Acids Facility.

In Vitro Construction of the Full Length cDNA Clone

Extension of the non full-length clone was accomplished by amplifying the missing region with site-specific primers using the polymerase chain reaction (PCR) as described by Frohman (1990). Substrate for PCR was cold first strand cDNA synthesized with reverse transcriptase using 1 μg of poly[A] RNA from air-adapted cells as template. Primer for the cDNA synthesis reaction was a pool of 14 bp oligonucleotides with random sequences. The upstream PCR primer was 5′-ACCCTGCGTCTAGAGTCATTA-3′ (base -44 to -22, Fig. 1) and the downstream primer was 5′-AATGTACAACTCAAGGGGAT-3′ (bases +530 to +549, Fig. 1). The upstream primer contained two bases not present in the published sequence (C at position -33 and A at position -31) to generate an *Xba I* restiction site (TCTAGA).

The 583 bp PCR product was gel purified, digested with the restriction endonucleases *Xba I* and *Bgl II*, and subcloned into the non full-length pBS-5 clone. Sequence analysis of the PCR-generated DNA fragment identified deletions at positions +8 and +9 (relative to the translation start site). The deletions were corrected using a double strand oligonucleotide made by annealing the oligonucleotides 5′-CATGGCGCGTACTGGCGC-3′ and 5′-CAGTACGCGC-3′. When these oligonucleotides anneal, over-hangs compatible with *Nco I* and *Hae II* result. These ends allowed bases -1 to +17, which surround the deletion, to be

replaced with wild type sequence. Obtaining the proper fragment of pBS-5 to accept the oligonucleotide was complicated by the presence of three other *Hae II* sites and two other *Nco I* site in pBS-5. Interference by the *Nco I* site at position +1955 and the *Hae II* site at position +1659 was avoided by using a subclone of pBS-5 lacking all of the sequence downstream of the unique *Bgl II* site. The *Nco I* site at position +123, containing a CAT codon for the HIS-42 residue, was eliminated by changing the codon to CAC using *in vitro* oligonucleotide-directed mutagenesis (Kunkel et al. 1987). This mutation is silent with regard to amino acid change. To generate and isolate the fragment with the necessary *Hae II* end, a partial digest of pBS-5 and polyacrylamide gel electrophoresis (Sambrook et al. 1989) were used.

RESULTS AND DISCUSSION

Isolation of Periplasmic CA cDNAs

The differential screening procedure proved successful in that seven perCA cDNA clones were isolated from $2X10^5$ plaques of the low CO_2-adapted library. The clones were identified as perCA by cross-hybridization to a partial genomic clone of *C. reinhardtii* perCA. The longest clone, pBS-5, was 1.5 kb while the limiting CO_2-induced transcript it hybridized to was 2 kb (no data shown), indicating the pBS-5 clone did not contain the entire coding region.

Nucleotide Sequence of the Periplasmic CA cDNA

During the time this work was in progress, Fukuzawa et al. (1990) reported cloning a full-length cDNA for perCA and later, Fujiwara et al. (1990) reported cloning two perCA genes, designated *CAH1* and *CAH2*. It was determined that the *CAH1* gene encodes a limiting CO_2-induced perCA while the *CAH2* gene encodes a much less abundant perCA, up-regulated by high CO_2 (Fujiwara et al. 1990). The published nucleotide sequence was used to verify that pBS-5 was the same as *CAH1*. The *CAH1* sequence was also used to choose sites to serve as priming locations to extend our clone to upstream of the translation start site using the polymerase chain reaction (PCR). The PCR priming sites are underlined on the nucleotide sequence of the full length cDNA (pCA)

shown in Figure 1. The upstream primer contained two nonhomologous nucleotides in order to generate a unique *Xba I* restriction site at this location and the downstream primer was designed so that the PCR product would contain the unique *Bgl II* restriction site at position +524 (relative to the translation start site). These steps were taken to facilitate subsequent cloning procedures. The positions of the 5′ terminus of the pBS-5 clone prior to extension by PCR, and a restriction endonuclease map of the full-length clone are shown in Figure 2.

Comparison of the *CAH1* sequence to that of the reconstructed full length clone showed that in the pCA clone, positions +8 and +9, corresponding to G and T nucleotides in the *CAH1* sequence, were deleted. Repeated attempts to replace the +8 and +9 nucleotides using a standard *in vitro* oligonucleotide-directed mutagenesis technique (Kunkel et al. 1987) were unsuccessful, but a strategy using a double stranded oligonucleotide to replace the surrounding region proved successful.

Although the cloning strategy was designed to ensure success at finding a perCA cDNA, it was expected to also find cDNAs for other low CO_2-induced transcripts. Polypeptides other than perCA, induced by exposure to limiting CO_2, have been reported (Manuel and Moroney 1988, Spalding and Jeffrey 1989), and since appearance of some of

Figure 1. Nucleotide sequence of periplasmic carbonic anhydrase cDNA clone pCA and deduced amino acid sequence of *C. reinhardtii* periplasmic carbonic anhydrase. Numbering of nucleotides is relative to the translation start site designated +1. Underlined regions identify the sites used for priming polymerase chain reaction. Bold-typed nucleotides within the upstream priming site are the nucleotides inserted to create an *Xba I* site. Part of the 3′ untranslated region was not determined (see also Fig. 2) and was excerpted from Fukuzawa et al. (1990)

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                    ACCCTGCGTCTAGAGTCATTACCTGCAACCCACTTGAACACC
                    -40       -30       -20       -10

 M  A  R  T  G  A  L  L  L  V  A  L  A  L  A  G  C  A  Q  A
ATGGCGCGTACTGGCGCTCTACTCCTGGTCGCGCTGGCGCTTGCGGGCTGCGCGCAGGCT
1        10        20        30        40        50        60

 C  I  Y  K  F  G  T  S  P  D  S  K  A  T  V  S  G  D  H  W
TGCATCTACAAGTTCGGCACGTCGCCGGACTCCAAGGCCACCGTTTCGGGTGATCACTGG
         70        80        90        100       110       120

 D  H  G  L  N  G  E  N  W  E  G  K  D  G  A  G  N  A  W  V
GACCACGGCCTCAACGGCGAGAACTGGGAGGGCAAGGACGGCGCAGGCAACGCCTGGGTT
        130       140       150       160       170       180

 C  K  T  G  R  K  Q  S  P  I  N  V  P  Q  Y  Q  V  L  D  G
TGCAAGACTGGCCGCAAGCAGTCGCCCATCAACGTGCCCCAGTACCAGGTCCTGGACGGG
        190       200       210       220       230       240

 K  G  S  K  I  A  N  G  L  Q  T  Q  W  S  Y  P  D  L  M  S
AAGGGTTCCAAGATTGCCAACGGCCTGCAGACCCAGTGGTCGTACCCTGACCTGATGTCC
        250       260       270       280       290       300

 N  G  T  S  V  Q  V  I  N  N  G  H  T  I  Q  V  Q  W  T  Y
AACGGCACCTCGGTCCAAGTCATCAACAACGGCCACACCATCCAGGTGCAGTGGACTTAC
        310       320       330       340       350       360

 N  Y  A  G  H  A  T  I  A  I  P  A  M  H  N  Q  T  N  R  I
AACTACGCCGGCCATGCCACCATCGCCATCCCTGCCATGCACAACCAGACCAACCGCATC
        370       380       390       400       410       420

 V  D  V  L  E  M  R  P  N  D  A  A  D  R  V  T  A  V  P  T
GTGGACGTGCTGGAGATGCGCCCCAACGACGCCGCCGACCGCGTGACTGCCGTGCCCACC
        430       440       450       460       470       480

 Q  F  H  F  H  S  T  S  E  H  L  L  A  G  K  I  Y  P  L  E
CAGTTCCACTTCCACTCCACCTCGGAGCACCTGCTGGCGGGCAAGATCTATCCCCTTGAG
        490       500       510       520       530       540

 L  H  I  V  H  Q  V  T  E  K  L  E  A  C  K  G  G  C  F  S
TTGCACATTGTGCACCAGGTGACTGAGAAGCTGGAGGCCTGCAAGGGCGGCTGCTTCAGC
        550       560       570       580       590       600

 V  T  G  I  L  F  Q  L  D  N  G  P  D  N  E  L  L  E  P  I
GTCACCGGCATCCTGTTCCAGCTCGACAACGGCCCCGATAACGAGCTGCTTGAGCCCATC
        610       620       630       640       650       660

 F  A  N  M  P  S  R  E  G  T  F  S  N  L  P  A  G  T  T  I
TTTGCGAACATGCCCTCGCGCGAGGGCACCTTCAGCAACCTGCCGGCGGGCACCACCATC
        670       680       690       700       710       720
```

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 K  L  G  E  L  L  P  S  D  R  D  Y  V  T  Y  E  G  S  L  T
AAGCTGGGTGAGCTGCTGCCCAGCGACCGCGACTACGTAACGTACGAGGGCAGCCTCACC
       730        740        750        760        770        780

 T  P  P  C  S  E  G  L  L  W  H  V  M  T  Q  P  Q  R  I  S
ACCCCGCCCTGCAGCGAGGGCCTGCTGTGGCACGTCATGACCCAGCCGCAGCGCATCAGC
       790        800        810        820        830        840

 F  G  Q  W  N  R  Y  R  L  A  V  G  L  K  E  C  N  S  T  E
TTCGGCCAGTGGAACCGCTACCGCCTGGCTGTGGGCCTGAAGGAGTGCAACTCCACGGAG
       850        860        870        880        890        900

 T  A  A  D  A  G  H  H  H  H  H  R  R  L  L  H  N  H  A  H
ACCGCCGCGGACGCCGGCCACCACCACCACCACCGCCGCCTGCTGCACAACCACGCGCAC
       910        920        930        940        950        960

 L  E  E  V  P  A  A  T  S  E  P  K  H  Y  F  R  R  V  M  L
CTGGAGGAGGTGCCTGCCGCCACCTCCGAGCCCAAGCACTACTTCCGCCGCGTGATGCTG
       970        980        990       1000       1010       1020

 A  E  S  A  N  P  D  A  Y  T  C  K  A  V  A  F  G  Q  N  F
GCCGAGTCCGCGAACCCCGATGCCTACACCTGCAAGGCCGTTGCCTTTGGCCAGAACTTC
      1030       1040       1050       1060       1070       1080

 R  N  P  Q  Y  A  N  G  R  T  I  K  L  A  R  Y  H  *
CGCAACCCCCAGTACGCCAACGGCCGCACCATCAAGCTGGCCCGCTATCACTAAACTTCC
      1090       1100       1110       1120       1130       1140

CAGTAGTTAGTCACGCTACCACCGTCGGCACGGCCAGCAGGCATTCCATTTTCCAGGCTT
TGCTTCACGGTTTGGTGTGTCATTCGATGGTGTTCTTGACGACCCGCGCTTGGCGGGCCT
TTCCAATTTTTTCCATAGTACACCGAAATAGTTCTGCGGTGCAGCACGCATACACACAGT
ACCGGACGGGCGGCGGGACCTCCTGTTTTCTCCTGACTAGTAAAGAAGTAAGGAAGGTAT
GGAGTTGGTTCCACGATGGGGCAGTCTGAGAGCGGAATAAAGTCAGTGGGCCGGACGTTG
TGGCGATGGATGGTAGTGAGGCAAGTAATACGTACGTAGAGGGCGTACGCGGGTAATAAC
GGGAACTTCGACAGCAATCGAGAGTGTCTGCACGCGAGACATTTGCGTACAGGGGAGGCA
CCGGTTCTCCTCGATGAGTGATCCGTACTTATGCAAGTTATATAAGGCTGGTGTGGGGCG
CTTCAGCACGGTATGGTTGCCAGCATGCACGGTCCGGCCTCTGTCTGGCTGGCTGGGTTG
TTGGCGGGCTGGCCTCATGCGCGCCGTCGCACATGCCGATCAATGCAGTTGCTCTCCAGT
AGCTGCAAGGCCTGGCTGGGCAATCCCATAGCCATGTCGAATGTGAAGCATTGTTTTCTT
GGAGATGGAGGACAGGAGACGCTGACCGGATGTTTTAAGACGTGCAGGATGTGGGGAGCG
AGGTAGCTACAACGGTGCAGTTGAGGCAGAGACGTGTACGACATGTAAGATGCCCATGGA
CAAAAAAAAAAAAAAAAAAAA
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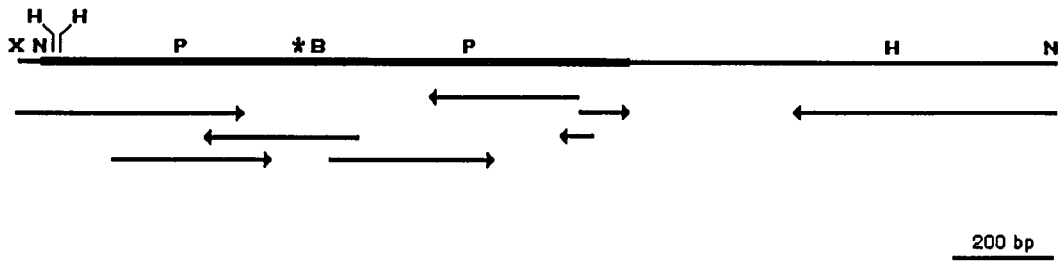

Figure 2. Restriction endonuclease map of the full-length periplasmic carbonic anhydrase cDNA clone pBS-5. The protein coding region is represented by a bold line. Arrows indicate boundaries and directions of over-lapping fragments sequenced. The sequence of each fragment was determined independently at least twice. Positions of recognition sites are shown for the following restriction endonucleases: *Bgl II*, (B); *Hae II*, (H); *Nco I*, (N); *Pst I*, (P); and *Xba I*, (X). "*" indicates the 5′ terminus of the original non full-length pBS-5 clone

these polypeptides corresponds to induction of the CO_2 concentrating mechanism (Spalding and Jeffrey 1989), it is believed at least some of them may function in CO_2 accumulation. Identification of cDNAs for these polypeptides would provide probes to study their regulation and possibly help assign functions. The differential screening approach to cloning should identify cDNAs for any low CO_2-induced polypeptide as long as its induction occurs at the level of transcript abundance.

One possible explanation for finding only perCA clones is that perCA might be the only limiting CO_2-induced polypeptide regulated at the level of transcript abundance. This seems unlikely though in view of evidence (Geraghty et al. 1990) that translatable mRNA for a 36 kD limiting CO_2 induced polypeptide is present in low CO_2-grown cells but not in high CO_2-grown cells. Another possibility for finding only perCA clones is that, since the cloning strategy enriched the library in perCA, other low CO_2-induced clones were artificially made rare. If this is the case, then to increase the chance of finding other clones, one could take measures to "subtract" perCA from the probes. For example, by adding saturating amounts of perCA to both probes, perCA would no longer appear as being differentially expressed and would not interfere with the identification of other, less abundant clones.

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PART III. OVER-EXPRESSION OF PERIPLASMIC CARBONIC ANHYDRASE BY A CELL WALL-DEFICIENT MUTANT OF *CHLAMYDOMONAS REINHARDTII*

ABSTRACT

Parallel inductions of the same magnitude occur in periplasmic carbonic anhydrase mRNA abundance, protein accumulation and activity when *Chlamydomonas reinhardtii* adapts to limiting environmental CO_2 concentrations. Cell wall-less strain CW-15 loses all its periplasmic carbonic anhydrase to the culture medium and over-produces periplasmic carbonic anhydrase mRNA and protein. These results suggest limiting CO_2-adapting *C. reinhardtii* cells possess a regulatory mechanism that both senses the amount of periplasmic carbonic anhydrase activity and controls its mRNA accumulation.

INTRODUCTION

Chlamydomonas reinhardtii and other unicellular algae possess a system to concentrate CO_2 intracellularly which enables very efficient photosynthesis in conditions of limiting external inorganic carbon (C_i; 0.03% CO_2 in air). *C. reinhardtii* cells exhibit high apparent affinity for CO_2, diminished O_2 inhibition of photosynthesis, reduced photorespiration and low CO_2 compensation concentration when grown in limiting C_i but not when grown with CO_2 enrichment (5% CO_2 in air).

Model systems proposed to explain intracellular accumulation of C_i from limiting external C_i sources (Moroney et al. 1987a, Spalding and Portis 1985), require C_i transport and an ability to interconvert CO_2 and bicarbonate both internal and external to the cell. Evidence for active C_i transport comes from analysis of a high CO_2 requiring mutant which has reduced C_i accumulation but wild type ability to interconvert C_i (Spalding et al. 1983a) and from direct measurement of active CO_2 transport in limiting-CO_2 grown *C. reinhardtii* cells (Sultemeyer et al. 1989).

Carbonic anhydrase (E.C.4.2.1.1; CA) is required to catalyze the equilibrium reaction between CO_2 and bicarbonate probably both intracellularly (Moroney et al. 1987a, Spalding and Portis 1985), either in the cytosol or the chloroplast or both (Sultemeyer et al. 1990), and

extracellularly in the periplasmic space (Moroney et al. 1987b, Sultemeyer et al. 1990). Internal CA catalyzes the dehydration of bicarbonate to supply CO_2 to ribulose bisphoshate carboxylase/oxygenase (Moroney et al. 1985, Moroney et al. 1987a, Spalding et al. 1983b) while periplasmic carbonic anhydrase (perCA), located in the periplasmic space or the cell wall, apparently is required to dehydrate bicarbonate to supply the preferred form of C_i for active transport (Sultemeyer et al. 1989). Intracellular CA activity has been demonstrated (Husic et al. 1989, Sultemeyer et al. 1990) but perCA activity accounts for nearly all (80-95%) of the CA activity in *C. reinhardtii* (Coleman et al. 1984, Kimpel et al. 1983, Yang et al. 1985).

Two different perCA genes have been reported in *C. reinhardtii* (Fujiwara et al. 1990). One gene (*CAH1*) is expressed in cells exposed to light and limiting external C_i and is highly expressed while the other gene (*CAH2*) appears to be down regulated by limiting external C_i but is not highly expressed under any condition (Fujiwara et al. 1990).

In *C. reinhardtii* accumulations of perCA mRNA and CA protein and induction of CA activity occur in the presence of light when high CO_2-cultured *C. reinhardtii* cells are switched to limiting CO_2 conditions (Dionisi-Sese et al. 1990, Fujiwara et al. 1990, Yang et al. 1985) In contrast,

when cells cultured in limiting CO_2 are returned to high CO_2 conditions, perCA mRNA accumulation, CA protein content and CA activity decrease (Bailly and Coleman 1988, Fujiwara et al. 1990, Yang et al. 1985).

In order to better understand the nature of perCA regulation by limiting environmental C_i concentrations the over-expression of perCA by a cell wall-deficient *C. reinhardtii* strain has been examined. *C. reinhardtii* with or without cell walls rapidly accumulate perCA transcript in response to limiting C_i, however, unlike a walled strain which exhibits a plateau in its expression of perCA after 24 hrs of limiting CO_2-growth, the wall-less strain accumulates approximately two-fold more transcript, protein and measured activity under the same circumstances. It is concluded that the retention of perCA activity in the periplasmic space indirectly controls the extent of perCA gene expression.

MATERIALS AND METHODS

Algal Strains and Culture Conditions

Chlamydomonas reinhardtii strains used were: cell wall-deficient mutant strain CW-15 (obtained from Dr. R. Togasaki, Indiana University) and wild-type strain 2137 (Spreitzer and Mets 1981). Cells were cultured as previously described (Geraghty et al. 1990) on an orbital shaker in minimal salts medium buffered for aeration with high CO_2 (5% CO_2 in air) or low CO_2 (0.03% CO_2 in air). High CO_2 cultures to be used in limiting CO_2 induction experiments were centrifuged and resuspended in minimal medium buffered for low CO_2 aeration. These cultures were then aerated with low CO_2 air for 90 min to flush the medium of elevated CO_2. The cultures were then shaken without aeration until being harvested. Cultures to be used for high CO_2 experiments were maintained with high CO_2 aeration and harvested in such a way as to minimize exposure to air and light.

RNA Isolation and PerCA Transcript Analysis

Total RNA was prepared from cells cultured with high CO_2-aeration or exposed to limiting CO_2 for 1, 4 or 24 hours. RNA (10 μg/lane) was electrophoresed through formaldehyde-containing agarose gels and transferred to nitrocellulose membrane (Fourney et al. 1988). A *CAH1* cDNA

identical in sequence to the reported *CAH1* cDNA (Fukuzawa et al. 1990) was isolated from a 4 hr, limiting CO_2 induced lamda-gt10 library by differential screening. The *CAH1* cDNA was radiolabelled and used to measure perCA mRNA accumulation on RNA gel blots following standard procedures (Sambrook et al. 1989). Quantitation of perCA mRNA accumulation was performed with scintillation counting of radioactivity bound to RNA blots.

Cell Fractionation, SDS-PAGE and Immunodetection

Cells cultured with high CO_2 or exposed to low CO_2 for 4, 8, 24 or 48 hours were fractionated (Geraghty et al. 1990) and soluble proteins were separated from membranes by centrifugation (48,000xg, 20 min). Extracellular proteins in CW-15 cultures were precipitated from the culture medium with ammonium sulfate (75% saturation) and were desalted using Amicon Centricon 30 concentrators. Excreted protein derived from the same volume of culture as the cellular proteins was combined with soluble protein samples for all CW-15 samples.

Equal protein (judged by silver staining) was electrophoresed through 12% polyacrylamide gels and transferred to nitrocellulose membrane (Geraghty et al. 1990). Detection of perCA was performed as previously described (Geraghty et al. 1990) using a polyclonal antibody kindly provided by Dr. David Husic (Husic et al. 1989). The

western blots were quantified by scanning densitometry. Because the antibody detected limiting CO_2-induced polypeptides of different molecular weights, densitometric scans for each sample were made of the two major perCA species (37 and 50 kDa) as well as all immunoreactive antigen. Both quantitations gave similar results. A dilution series was used to demonstrate that, in the range of signal intensity being measured, the measured response was linear.

Carbonic Anhydrase Activity Assay

CA activity was measured by the electrometric method (Yagawa et al. 1986) for washed and disrupted cells and for extracellular protein found in the culture medium. Preparation of samples for measuring CA activity in cellular and in supernatant fractions were done simultaneously from 6 mL of the same culture. Protein in culture supernatant was concentrated from the medium with Amicon Centricon 30 concentrators and resuspended in buffer. CA activity units are expressed on a per mg chlorophyll basis.

RESULTS AND DISCUSSION

Carbonic Anhydrase Activity

CA activity was measured separately for washed and broken cells and for protein excreted into the culture medium (Table 1). As has been reported (Kimpel et al. 1983), cell wall-deficient mutant CW-15 lost essentially all its CA activity to the culture medium while activity in cells with an intact cell wall (strain 2137) was associated only with the cells. After adaptation to limiting CO_2 for 24 hours, nearly twice as much CA activity was associated with CW-15 culture medium than with 2137 cells and after 48 hours of limiting CO_2 growth, CW-15 cells produced almost three times as much CA activity. From 24 to 48 hours of limiting CO_2 adaptation, CA activity in 2137 cells did not increase, suggesting that by 24 hours (but possibly much earlier) the cells had synthesized sufficient activity to supply the required function. In CW-15 cells however, activity nearly doubled over this time period (Table 1 and Kimpel et al. 1983) and may continue to increase after 48 hours. This finding suggests cells cultured under limiting CO_2 require a minimum level of CA activity and that a mechanism exists to achieve and maintain the level of activity required. It appears the control mechanism may be affected somehow by CA activity in the periplasmic space.

Table 1. Quantitation of periplasmic carbonic anhydrase expression by *C. reinhardtii* strains 2137 and CW-15

	Growth in limiting CO_2[a] (hr)				
	0	1	4	24	48
Activity[b]					
2137					
Cells	n.d.[c]	-[d]	-	64±4	67±3
Supernatant	n.d.	-	-	7±6	3±1
CW-15					
Cells	n.d.	-	-	4±3	3±2
Supernatant	n.d.	-	-	108±3	186±18
Protein[e]					
2137	17	-	27	180	187
CW-15	15	-	34	475	816
mRNA[f]					
2137	n.d.	3.5	54.7	40	-
CW-15	5.6	39.3	112.5	96	-

[a]Cultures were harvested after being grown with 5% CO_2 (0 hr) or with 0.03% CO_2 for 1, 4, 24, or 48 hours.

[b]Values of CA activity in units/mg chl are the average of four assays from a total of two experiments.

[c]None detected.

[d]Not determined.

[e]Densitometry (arbitrary units) of immunodetected CA.

[f]Radioactivity ($x10^2$ cpm) bound to perCA transcript.

Accumulation of Carbonic Anhydrase Protein

CA protein from cell extracts and culture supernatant was combined and detected on western blots with antiserum against perCA. Major reactive polypeptides were the 37 kD perCA large subunit monomer and a 50 kD polypeptide. Both the 37 and 50 kD polypeptides resolve into two bands (or one broad band). Alternate glycosylation patterns have been proposed to explain the resolution of the 37 kD large subunit monomer into two bands (Husic et al. 1989). The identity of the 50 kD polypeptide is not clear since 50 kD is too small for large subunit dimers and aggregation of large and small subunits seems unlikely because the subunits dissociate readily with DTT treatment (Husic et al. 1991). Interestingly, the 50 kD and 37 kD polypeptides appear to be present in near equimolar amounts in limiting CO_2 adapting CW-15, but not 2137 cells. A minor polypeptide of >100 kD evident in panel B of Figure 1 may be the 110 kD form of CA found both in the chloroplast stroma and whole cell fractions by Husic et al. (1989). The immunologically related polypeptides in the range of 70 and 90 kD (panel B Figure 1) may be dimers and trimers, respectively of the 37 kD CA subunit. That all the above mentioned reactive polypeptides are forms of CA is suggested by their gradual appearance in the time-course of adaptation to limiting CO_2 and by strong cross-reactivity to the antibody.

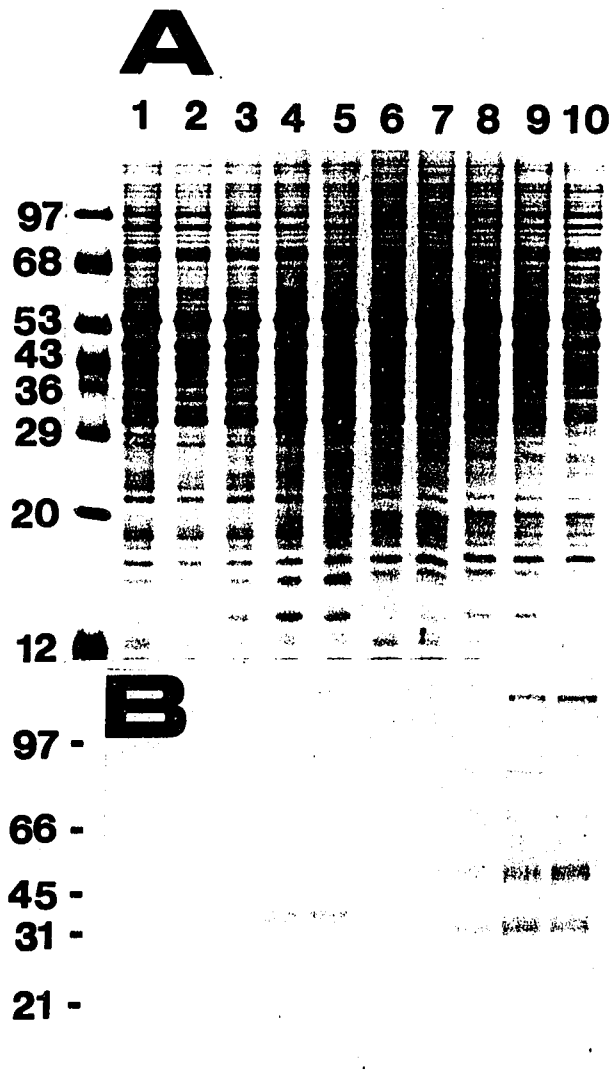

Figure 1. Limiting CO_2-induced accumulation of perCA protein in 2137 cells and in CW-15 cells and culture supernatant. A) SDS-PAGE and silver staining of protein samples from 2137 cells (lanes 1-5) and CW-15 cells (lanes 6-10) aerated with elevated (5%) CO_2 (lanes 1 and 6) or with limiting CO_2 (0.03%) for 4 hr (lanes 2 and 7), 8 hr (lanes 3 and 8), 24 hr (lanes 4 and 9) or 48 hr (lanes 5 and 10). B) Immunodetection using perCA antiserum against identical samples as in the photographed gel following transfer to nitrocellulose

Westen blot quantitation demonstrated that for limiting CO_2-adapting CW-15 cells, the two-fold over expression of CA activity (in comparison to 2137 cells) resulted from parallel elevated CA protein content. Similar quantitative results were obtained when total immunoreactive antigen was measured and when the quantitation was limited to the 37 and 50 kD polypeptides. By 24 hours of limiting CO_2 adaptation, CW-15 cells had excreted more than twice the amount of CA protein than had accumulated in 2137 cells and more than four times the amount after 48 hours. As was the case with CA activity measurements, no significant increase occurred from 24 to 48 hours in the amount of CA protein accumulated in 2137 cells, however CA protein content almost doubled in CW-15 cell culture medium over this period (Table 1 and Figure 1) and may accumulate to higher levels beyond 48 hrs.

Accumulation of Periplasmic Carbonic Anhydrase mRNA

Detection of perCA mRNA on Northern blots with a perCA cDNA was used to monitor perCA mRNA abundance in cells adapting to limiting CO_2. 2137 cells achieved maximum perCA mRNA accumulation between 1 and 4 hours of limiting CO_2 exposure, but by 24 hours, accumulation was reversed slightly. In contrast, CW-15 cells accumulated perCA mRNA earlier in response to limiting external CO_2 concentration and to a greater extent at maximum transcript accumulation.

The qualitative difference between perCA accumulation

in the two strains has been reported (Spalding et al. 1991), however, in the absence of quantitation, the degree of difference was over estimated. As for CA activity and protein accumulation, perCA mRNA was about twice as abundant in CW-15 cells compared to 2137 cells after 24 hours of low CO_2 adaptation (Table 1 and Figure 2). Taken together, these measurements indicate increased perCA transcript abundance is responsible for over-expression of perCA at the protein and activity levels in CW-15.

It is interesting that in high CO_2 cultured CW-15 cells a detectable level of perCA mRNA accumulated. This could be *CAH2* mRNA (Fujiwara et al. 1990) since the *CAH1* cDNA probe will detect both carbonic anhydrase mRNAs. The possibility that *CAH2* mRNA is detectable in CW-15 cells (lane 5, Fig. 2) but not in 2137 cells (lane 1, Fig. 2), might suggest that CW-15 cells over-express *CAH2* as well as *CAH1*.
We have found that when cells are adapting to low CO_2, parallel increases of similar magnitude occur in CA activity, protein accumulation and mRNA abundance. The lack of an intact cell wall in CW-15 prevents retention of extracellular CA in the periplasmic space (Kimpel et al. 1983) which appears to result in its over-production. Measuring perCA over production by a cell wall-deficient strain has provided evidence which suggests *Chlamydomonas*

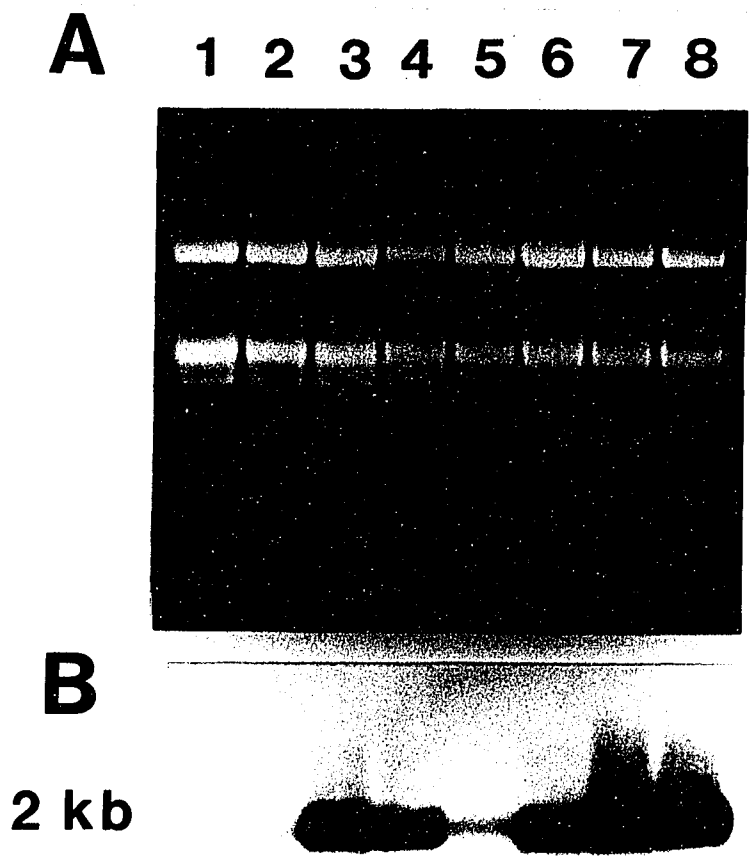

Figure 2. Limiting CO_2-induced accumulation of perCA mRNA in 2137 and CW-15 cells. Cellular RNA from 2137 (lanes 1-4) and CW-15 (lanes 5-8) was separated by formaldehyde-agarose electrophoresis and photographed (panel A), then transferred to nitrocellulose and probed with a radiolabelled *CAH1* cDNA (panel B). Cells were aerated with elevated (5%) CO_2 (lanes 1 and 5) or with limiting (0.03%) CO_2 for 1 hr (lanes 2 and 6), 4 hr (lanes 3 and 7) or 24 hr (lanes 4 and 8)

cells posses a mechanism that can both perceive the amount of CA activity in the periplasmic space and affect CA activity by modulating perCA mRNA accumulation.

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PART IV. EXPRESSION OF THE *CAH1* AND *CABII-1* GENES IN *CHLAMYDOMONAS REINHARDTII* DURING INDUCTION OF THE CO_2 CONCENTRATING MECHANISM: STUDIES USING A MUTANT DEFICIENT IN THE CO_2 CONCENTRATING MECHANISM

ABSTRACT

Upon exposure to limiting external inorganic carbon (CO_2 + HCO_3^-), *Chlamydomonas reinhardtii* cells induce a system to transport and accumulate CO_2. Operation of this CO_2 concentrating mechanism creates a high internal CO_2 concentration that saturates photosynthesis. As a result, the oxygenase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase is inhibited by competition with CO_2, and the flow of carbon through the photorespiratory pathway is thus diminished. Several changes in protein and gene expression have previously been documented in cells switched from high to low external inorganic carbon concentrations.

This study demonstrates that a mutant deficient in the CO_2 concentrating mechanism fails to induce accumulation of periplasmic carbonic anhydrase mRNA, and surprisingly, that the mRNA for one of the light harvesting chlorophyll binding proteins changes in abundance in wild type cells in response to limiting CO_2. Because this change is absent from the mutant, it was concluded that this is a response induced by limiting CO_2.

INTRODUCTION

Chlamydomonas reinhardtii and other unicellular algae acquire an ability to actively accumulate inorganic carbon (C_i) under conditions of limiting external C_i (for reviews see Badger 1987, Spalding 1989). Energy-requiring C_i transport and carbonic anhydrase (CA) activity are induced by CO_2 limitation, resulting in a high intracellular CO_2 concentration. The effect of the CO_2 concentrating mechanism on photosynthesis is to reduce photorespiration and O_2 inhibition of photosynthesis by competitively inhibiting the oxygenase activity of the bifunctional enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco). Owing to their ability to accumulate C_i against a concentration gradient, low CO_2-grown cells have a much higher apparent affinity for CO_2 in photosynthesis than either high CO_2-grown cells or isolated *C. reinhardtii* rubisco.

Evidence indicates that CA activity external to the cell, either in the periplasmic space or associated with the cell wall, supplies CO_2 from HCO_3^- at alkaline pH (Moroney et al. 1985), and that cells selectively remove CO_2 from their culture medium (Moroney et al. 1985, Sultemeyer et al. 1989). Periplasmic carbonic anhydrase (perCA), coded for by the *CAH1* gene (Fukuzawa et al. 1990) is the only component of the CO_2 concentrating mechanism physically identified

thus far. Analyses of *C. reinhardtii* mutants (Moroney et al. 1986, Spalding et al. 1983c) that require elevated CO_2 concentration for photoautotrophic growth have been used to delineate three other genes likely to code for components of the CO_2 concentrating mechanism, however, the components themselves remain uncharacterized. Mutations that result in deficiencies either in C_i transport (Spalding et al. 1983b) or in internal CA activity (Spalding et al. 1983a), identify loci that are essential for operation of the CO_2 concentrating mechanism.

Another locus, *cia-5*, has been identified which, when mutated, results in the absence of all known components and responses normally associated with the CO_2 concentrating mechanism (Moroney et al., 1989). Moroney et al. (1989) demonstrated that, when switched from high to low CO_2 concentrations, cells with a lesion at the *cia-5* locus do not develop higher affinity for CO_2; are unable to accumulate C_i to a level higher than that which can be attributed to simple diffusion; probably lack internal CA activity; fail to induce three limiting CO_2-induced polypeptides putatively involved in CO_2 accumulation; and lack induction of perCA at the level of protein accumulation, and therefore, have no measurable CA activity. In addition, Spalding et al. (1991) have reported the absence of detectable translatable mRNA for a 36 kD limiting

CO_2-induced polypeptide in CIA-5 cells. The limiting CO_2-induced transient decline in rubisco small subunit synthesis observed in wild type cells (Coleman and Grossman 1984, Spalding and Jeffrey 1989) also is missing from CIA-5 cells (Moroney et al. 1989). An explanation for decreased small subunit synthesis due to exposure to limiting CO_2 is lacking, but the level of control has been shown to be at translation (Part I, this dissertation). Upon exposure to limiting CO_2, CIA-5 cells also do not exhibit the transient increase in activity of phosphoglycolate phosphatase (Spalding et al., 1991), the first committed enzyme in the photorespiratory pathway. A temporary increase in this activity in wild type cells is thought to enable the cell to metabolize the elevated level of phosphoglycolate resulting from higher photorespiratory activity in the few hours following transfer to limiting CO_2 before the CO_2 concentrating mechanism becomes operational.

In view of the wide range of responses missing in the CIA-5 mutant, Moroney et al. (1989) and Spalding et al. (1991) have speculated that this locus may define a regulatory component of the CO_2 concentrating mechanism; perhaps the signal responsible for adaptation to limiting CO_2 or a signal transducing function.

The findings presented in this report indicate that transcripts for periplasmic carbonic anhydrase encoded by

the *CAH1* gene and for a light harvesting chlorophyll binding protein (LHCP) encoded by the *cabII-1* gene, both respond to limiting CO_2 with changes in abundance. Substantial changes for these transcripts in wild type cells but not in CIA-5 cells indicate adaptation to limiting CO_2 probably affects these responses. These findings further demonstrate the extent of responses affected by the *cia-5* locus.

MATERIALS AND METHODS

Algal Strains and Culture Conditions

Chlamydomonas reinhardtii wild-type strain 2137 (Spreitzer and Mets 1981) and high CO_2-requiring mutant CIA-5 (Moroney et al. 1989; obtained from Dr. J. Moroney, Louisiana State University) were cultured in the minimal salts media described by Geraghty et al. (1990). Cells were cultured photoautotrophically in liquid medium on a gyratory shaker and were either constantly aerated with 5% CO_2 in air (high CO_2-grown cells) or grown without aeration (fully air-adapted cells). For analysis of limiting CO_2-induced responses, cells cultured with elevated CO_2 were switched to aeration with air level CO_2 (0.03% CO_2 in air) for 1, 2, 4, 8, 12 or 24 hours.

RNA Isolation and Northern Analysis

Total RNA was isolated as previously described (Geraghty et al. 1990). Total RNA (10 μg per lane) was electrophoresed in formaldehyde-containing agarose gels and transferred to nitrocellulose sheets. RNA sample preparation, gel composition, electrophoresis buffers and capillary transfer of RNA to nitrocellulose sheets were performed as described by Fourney et al. (1988). Standard methods were used for hybridization and autoradiography. Quantitation of *cabII-1* mRNA abundance was performed by

liquid scintillation spectroscopy of bands excized from the gel blot.

Gene-Specific Probes

A 1.5 kilobase periplasmic CA cDNA (pBS-5) was obtained by differential screening of a cDNA library made with RNA from air-adapted cells. Radiolabeled, reverse transcribed poly[A] RNA from either CO_2-enriched or air-adapted cells was used to probe duplicate samples of the library. Plaques that hybridized exclusively to the air-adapted probe were analyzed further. That pBS-5 is periplasmic CA has been verified by comparison of its sequence to the published *CAH1* cDNA (Fukuzawa et al. 1990). The 1.2 kilobase *cabII-1* transcript was measured using the cDNA clone pHS16 obtained from Dr. S. Howell (Shepherd et al. 1983).

RESULTS AND DISCUSSION

Steady-state mRNA levels were measured over the time course of adaptation to limiting CO_2 for perCA, encoded by *CAH1*; and for an LHCP, encoded by *cabII-1*. Comparison of the CIA-5 mutant to a wild type strain with regard to these parameters is a way to identify additional changes in *C. reinhardtii* cells developing the CO_2 concentrating mechanism.

Accumulation of CAH1 mRNA

Wild type *C. reinhardtii* cells switched from high to low concentrations of CO_2 accumulated *CAH1* mRNA whereas cells cultured continuously with elevated CO_2 did not (Fig. 1A). Unlike wild type cells, CIA-5 cells did not accumulate detectable *CAH1* mRNA when exposed to limiting CO_2 (Fig. 1A). Absence of *CAH1* mRNA in CIA-5 demonstrates that perCA protein deficiency reported for this mutant (Moroney et al. 1898) is controlled by transcript abundance.

It is well established that external CA activity is a component of C_i accumulation (Badger et al. 1980, Coleman et al. 1984, Moroney et al. 1985) and that its induction is controlled at the level of transcription (Bailly and Coleman 1988, Fukuzawa et al. 1990). The role of perCA in C_i accumulation is demonstrated by studies using inhibitors of CA in conditions of limiting external C_i (Moroney et al.

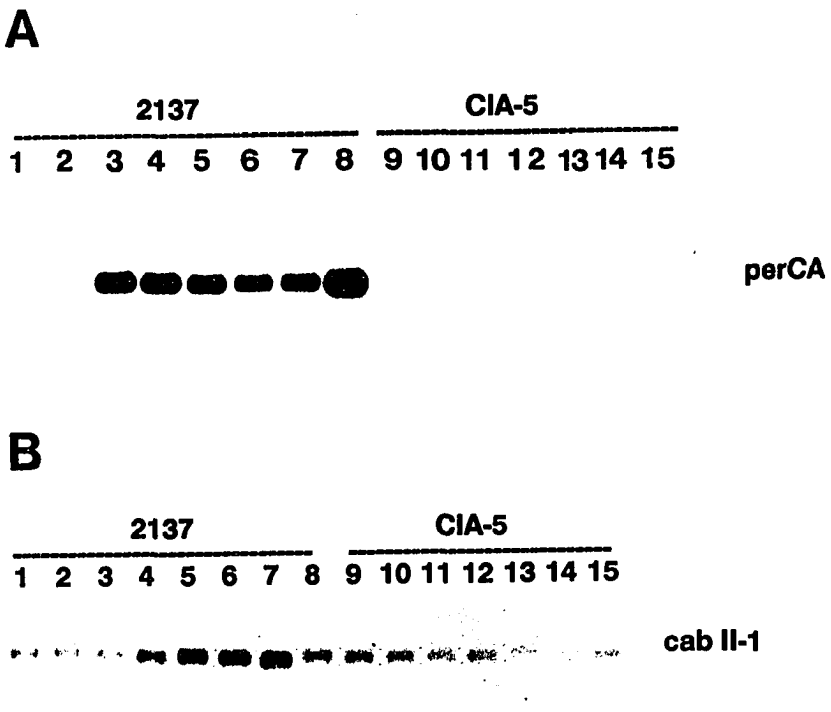

Figure 1. Autoradiogram showing the time course of limiting CO_2-induced accumulation of *CAH1* mRNA (panel A) and *cabII-1* mRNA (panel B). Equal amounts of RNA from *C. reinhardtii* wild type strain 2137 (lanes 1-8) and mutant strain CIA-5 (lanes 9-15) were probed with gene-specific probes. Times of exposure to limiting CO_2 were 0 hr (lanes 1 and 9), 1 hr (lanes 2 and 10), 2 hr (lanes 3 and 11), 4 hr (lanes 4 and 12), 8 hr (lanes 5 and 13), 12 hr (lanes 6 and 14) and 24 hr (lanes 7 and14). Lane 8 is from fully air-adapted wild type cells

1985). Experiments done at alkaline pH in the presence or absence of inhibitors demonstrate that CA-catalyzed dehydration of HCO_3^- to CO_2 at the cells external surface is required to sustain a high photosynthetic rate and C_i accumulation. However, whether or not external CA activity is essential for high rates of photoautotrophic growth at a neutral pH is yet to be determined.

Accumulation of CabII-1 mRNA

Wild type cells exposed to limiting CO_2 for 4, 8, 12 and 24 hours accumulated increasingly more *cabII-1* mRNA, whereas CIA-5 cells did not (Fig. 1B). Quantitation of wild type *cabII-1* transcript abundance revealed that cells adapted to low CO_2 for 4 hr, 8 hr, 12 hr, or 24 hr accumulated 1.6, 1.8, 2.3 and 2.6 fold more *cabII-1* mRNA than high CO_2-grown cells, respectively. No significant increase or decrease was measured for CIA-5 cells. These findings are somewhat of a surprise in view of the role of LHCPs as apoproteins to bind light harvesting chlorophyll a and b in the chlorophyll-protein complex of photosystem II; a function which would seem not to be involved in C_i accumulation. One possible explanation for increased *cabII-1* mRNA accumulation in wild type cells exposed to limiting CO_2 is that a shock, resulting from the change in CO_2 concentration, may have synchronized the cell cycles of these cells. Synchronous cultures would be expected to

accumulate more *cabII-1* mRNA at the time in the diurnal cycle corresponding to maximum expression than a nonsynchronous culture would accumulate at anytime. *CabII-1* transcript accumulation has been shown to vary in a diurnal manner in synchronous *C. reinhardtii* cultures (Shepherd et al. 1983), and to be light regulated (Imbault et al. 1988, Kindle 1987, Shephard et al. 1983). There is no other indication, however, that setting of the cell cycles actually happens as a result of changing CO_2 concentrations.

It might also be thought that increased growth and division rates for wild type cells may alone account for the gradual increase in *cabII-1* mRNA abundance over the time course, and because the CIA-5 mutant cannot induce the system to accumulate CO_2, its growth and division rates would be inhibited in limiting CO_2 so its *cabII-1* mRNA levels would remain unchanged. This possibility seems unlikely though because 24 hr limiting CO_2-adapted cells accumulated more *cabII-1* mRNA than either high CO_2-grown cells or fully air-adapted cells, even though growth and division rates in the three cultures were all approximately the same.

The correlation between exposure to CO_2 limitation and *cabII-1* mRNA accumulation in wild type cells, and the absence of this response in CIA-5 cells, together suggest that *cabII-1* mRNA abundance is regulated in limiting CO_2 by

the same signal which is responsible for inducing other components of the CO_2 concentrating mechanism.

It has previously been established that the *cia-5* locus in *C. reinhardtii* defines a component with a wide array of regulatory capabilities in cells undergoing adaptation to limiting CO_2: none of the components or responses identified as being required for, or correlated with, the CO_2 concentrating mechanism are present in cells when this locus is mutated (Moroney et al. 1989, Spalding et al. 1991). This report demonstrates that the CIA-5 mutant strain is deficient in two additional responses normally induced by exposure to limiting CO_2. The findings related to *CAH1* mRNA induction are not surprising in view of the essential role for CA activity in C_i accumulation. On the other hand, evidence that *cabII-1* transcript abundance is affected by exposure to limiting CO_2 is surprising because LHCPs would not be expected to be involved in the ability to accumulate CO_2.

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SUMMARY

Unlike terrestrial C3 type plants, unicellular algae such as *Chlamydomonas reinhardtii* have developed a relatively simple mechanism to reduce photorespiration. *C. reinhardtii* cells grown in CO_2-limiting conditions reduce competitive inhibition of photosynthesis by oxygen by raising the intracellular CO_2 concentration through activities of a CO_2 concentrating mechanism and carbonic anhydrase. The mechanism is induced in cells exposed to low CO_2 and is absent in cells grown with high CO_2.

Because the microalgal CO_2 concentrating mechanism is probably simpler than higher plant CO_2 concentrating systems (only a few genes may be required), it may someday be possible to introduce it into C3 crop species to enable them to photosynthesize more efficiently. For this reason it is important to better understand the CO_2-dependent changes in gene expression which enable *C. reinhardtii* cells to accumulate CO_2 and avoid O_2-inhibition of photosynthesis. In this study, the inducible nature of the mechanism was utilized to identify and characterize changes in gene expression coincidental with acquisition of the ability to concentrate CO_2.

One part of the study describes the cloning of a component of the CO_2 concentrating mechanism. A cDNA clone for the limiting CO_2-induced periplasmic carbonic anhydrase

(perCA) was identified using a strategy that relied on the difference in perCA transcript abundance between cells cultured with elevated CO_2 and those cultured with limiting CO_2. The perCA over-expressing strain CW-15 was used to construct the cDNA library in hopes that this would work to increase the relative difference in perCA transcript abundance. Several perCA cDNA clones were isolated and one full length clone was generated using the polymerase chain reaction. The nucleotide sequence of this clone was determined and an open reading frame coding for 378 amino acids was found. Having this cDNA permitted the qualitative and quantitative measures of perCA transcript accumulation as reported in Parts III and IV.

Another part of the study dealt with the observation that synthesis of ribulose 1,5-bisphosphate carboxylase/oxygenase large (L) and small (S) subunits is diminished in cells inducing the CO_2 concentrating mechanism. Analyses of rubisco gene expression in inducing cells demonstrated that decreased accumulation of newly made subunits resulted from specific and coordinate down-regulation of translation. No contribution to diminished subunit synthesis by regulation of transcript pool size or by post-translational turn-over was found.

It was speculated that reduced L and S synthesis might be a consequence of a limited supply of resources within the

cell for building macromolecules, for example, the rubisco holoenzyme. Demand for resources to synthesize components of the CO_2 concentrating mechanism might interfere with production of expendable macromolecules; and production of rubisco subunits may be expendable for short periods owing to the stability of the holoenzyme.

While the rubisco study showed that translation can be the target of regulation during induction, quantitation of expression of *CAH1* (which codes for periplasmic carbonic anhydrase) in the cell wall-deficient mutant CW-15 provided evidence for transcriptional regulation. The CW-15 mutant had previously been shown to release essentially all its perCA to the culture medium and, compared with a walled strain, two-fold more CA enzyme activity was measured in CW-15 cultures after growth in limiting CO_2 for 24 hours. Consistent with higher measured activity, CA protein and transcript also over-accumulated. At each level of expression the increases were of the same magnitude. These results show that retention of perCA activity in the periplasmic space indirectly controls the extent of perCA transcript accumulation, demonstrating transcriptional regulation.

Further evidence for transcriptional regulation during induction of the CO_2 concentrating mechanism comes from analysis of a mutant deficient in the CO_2 concentrating

mechanism. CIA-5 mutant cells were shown to lack induction of perCA transcript accumulation and to fail to exhibit increased accumulation of *cabII-1* transcript in response to limiting CO_2. Since this mutant had previously been shown to lack all components of the CO_2 concentrating mechanism, it was not unexpected to find the absence of perCA transcript accumulation. However, it was surprising to find that *cabII-1* transcript abundance was controlled by CO_2 concentration. The *cabII-1* gene product functions in photosystem II to bind light harvesting chlorophyll, and so it is not immediately clear why abundance of its transcript would be affected by CO_2 concentration. The findings for the CIA-5 mutant further demonstrate the wide range of responses affected by exposure to CO_2-limitation in *C. reinhardtii* cells, and suggests that the requirements for induction of the CO_2 concentrating mechanism are more numerous than previously believed.

Together, the results of this study demonstrate that regulation of gene expression during induction is exerted by transcriptional (*CAH1* and *cabII-1*) as well as by translational (*rbc*S and *rbc*L) mechanisms.

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