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Light-Induced Carbonic Anhydrase Expression in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The effect of external inorganic carbon concentration and light on carbonic anhydrase (CA) protein accumulation and steady-state mRNA levels were examined in *Chlamydomonas reinhardtii*. When photoautotrophically grown high-CO_2 cells were transferred to low-CO_2 conditions, they exhibited a significant accumulation of the 2.0-kilobase CA transcript after 1 hour with the maximum level reached after 2 hours. An increase in the accumulation of the 37-kilodalton CA monomer was observed after 2-hour exposure to air. Cells allowed to adapt to air levels of CO_2 in the dark showed neither an increase in CA mRNA abundance nor in the accumulation of the enzyme. Similarly, addition of 10 micromole 3-(3,4-dichlorophenyl)-1,1-dimethylurea immediately after transferring high-CO_2 cells to low-CO_2 condition did not cause an increase in CA transcript abundance and enzyme accumulation, suggesting that photosynthesis is absolutely required in the regulation of CA transcript abundance. In addition to the photosynthesis-dependent process, a blue light stimulated mechanism is also involved in CA transcript regulation. Experiments with transcription inhibitors confirmed the notion that the gene for carbonic anhydrase enzyme is encoded by the nuclear genome and that the induction of the enzyme depends heavily on the transcription of the CA gene.

It has been established that *Chlamydomonas reinhardtii* cells grown in ordinary air containing 0.04% (v/v) CO_2 (low-CO_2 cells) are more efficient in the utilization of DIC[3] (CO_2 and HCO_3^-) for photosynthesis than cells grown in air enriched with 5% (v/v) CO_2 (high-CO_2 cells) (2, 4). The high affinity for DIC utilization in low-CO_2 cells is explained by carbonic anhydrase (CA; EC 4.2.1.1) which catalyzes the reversible hydration of CO_2, and the existence of CO_2-concentrating mechanism, which is achieved via active DIC transporters (1, 18). Most of the enzyme activity observed in low-CO_2 cells of *C. reinhardtii* is located outside the plasmalemma, either in the periplasmic space or attached to the cell wall (13, 31). The periplasmic CA has been shown to be important in the supply to the cells of free CO_2 from the dehydration of HCO_3^- (1, 25), and has been identified to be a glycoprotein with a subunit molecular mass estimated at 35 to 37 kD (6, 29, 32). The induction of CA activity under low-CO_2 conditions was shown to be inhibited by the protein translation inhibitor, cycloheximide (22, 24, 32), suggesting that the enzyme was synthesized *de novo* when the CO_2 concentration was lowered. Subsequently, evidence was provided confirming that the increase in CA activity paralleled the increase in CA protein (32). Isolation of poly(A)$^+$ RNA from *C. reinhardtii* cells and analysis of the *in vitro* translation products showed that the synthesis of the precursor polypeptide was greater with mRNA from low-CO_2 cells than from high-CO_2 cells, indicating that the induction of CA in *C. reinhardtii* is regulated at a level before translation (7, 28). The biosynthesis and intracellular processing of this enzyme was then studied by Toguri *et al.* (29) in the wall-less mutant of *C. reinhardtii* cw-15 which secretes CA into the culture medium.

It was shown that in addition to a lowering of CO_2 concentration, light is also required for CA induction in *C. reinhardtii* (23, 24). We have shown that light exerts its control on CA activity of *C. reinhardtii* through photosynthesis as well as through a photosynthesis-independent blue light-requiring process (9, 10). A suggestion on the possible regulatory sites of these two processes on CA biosynthesis was made based from the results of inhibitor addition on CA activity. To confirm the roles of light on CA induction, in addition to a lowering of CO_2 concentration to air level, the effects of light on CA protein accumulation and CA transcript abundance were investigated.

MATERIALS AND METHODS

Algal Strain and Culture Condition

Chlamydomonas reinhardtii Dangeard C-9 *mt*$^-$ (Center for Microbial and Microalgal Research of the Institute of Applied Microbiology, University of Tokyo) was grown as described previously in 3/10 high salt concentration medium under continuous illumination (18 W/m^2) at 30°C with constant bubbling of 5% CO_2-enriched air (9). To induce CA, high-CO_2 cells were resuspended in fresh culture medium at the density of 1 ± 0.2 mL packed cell volume per liter and the bubbling gas changed to ordinary air (0.04% CO_2). To study the time course of CA induction and to examine the effects of different inhibitors, the cells were illuminated from one side with banks of fluorescent lamps at an energy fluence rate

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[3] Abbreviations: DIC, dissolved inorganic carbon; CA, carbonic anhydrase; high-CO_2 cells, algal cells grown in air enriched with 5% CO_2 (v/v); low-CO_2 cells, algal cells grown in air containing 0.04% CO_2 (v/v); BPB, bromophenol blue; kb, kilobase.

at 18 W/m^2. To study the blue and red light effects on CA induction, the illumination procedure was the same as that described previously (10). Cells were illuminated on one side with red light (620–680 nm; 4 or 5.3 W/m^2) obtained by placing a glass filter (Hoya Glass Co., Tokyo) and infrared reflecting filter (Vacuum Optics Co., Tokyo) between the algal suspension and a slide projector equipped with 150 W halogen lamp (Color Cabin III, Tokyo). On the other side, the cell suspension was illuminated with blue light (460 nm; 1.3 W/m^2) obtained by the combined use of interference filter with half-band width of 10 to 20 nm and appropriate color filter. Thermal radiation was eliminated by an infrared reflecting glass filter inserted between the algal suspension and the light source, 5-kW xenon lamp (Ushio Electric Inc., Tokyo). In the case of white light (380–680 nm) illumination, only infrared reflecting glass filter was placed between the algal suspension and the halogen lamp source.

Preparation of Antibodies

Purified *C. reinhardtii* CA was chemically deglycosylated by trifluoromethanesulfonic acid treatment following the procedure previously described (29). Antiserum against this deglycosylated CA was then prepared essentially as described by Yang *et al.* (32). To obtain an antibody specific only to CA, the obtained antiserum against deglycosylated CA was further purified by passing the antiserum over an Affi-Gel 10 (Bio-Rad) column to which authentic CA repurified by anion-exchange HPLC (TSK gel DEAE-5PW) had been bound.

SDS-PAGE and Immunoblotting

Cells continuously bubbled with 5% CO_2 or allowed to adapt to air levels of CO_2 for specific periods of time at high light intensity illumination (18 W/m^2) in the presence or absence of DCMU (10 μM) or with or without additional red or blue light illumination were harvested by centrifugation, broken by sonication at 4°C and centrifuged briefly. The total soluble proteins in the supernatant were precipitated and suspended in 50 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (w/v) sucrose, 50 mM DTT, and 0.002% (w/v) BPB. Prior to electrophoresis, proteins were solubilized by incubating at 60°C for 10 min and spun down to remove insoluble materials. SDS-PAGE was carried out in 1-mm thick 12.5% (w/v) polyacrylamide gels as described previously (15) but with twice the concentration of Tris and glycine in the reservoir buffer as recommended by Piccioni *et al.* (20). The gel was calibrated with mol wt standard proteins (Dalton Mark VII-L, Sigma). Separated polypeptides on the SDS-polyacrylamide gel were then electrotransferred to polyvinylidene difluoride filter (Immobilon PVDF, Millipore) in 25 mM Tris (pH 11) as the electrode solution at 100 V for 2 h at 4°C. The Western blot was pretreated with 10 mM Na-phosphate buffer (pH 7.2) containing 0.05% (v/v) Tween 20 and 150 mM NaCl (Tween-PBS) (8) for 1 h at room temperature. After an overnight incubation with the affinity purified anti-deglycosylated CA antibody at 4°C, the blot was successively washed with Tween-PBS for several times to remove unbound antibody. Immunoreactive bands were then detected with *Staphylococcus aureus* ^{125}I-labeled protein A (New England Nuclear) (16). After 1 h incubation at room temperature, nonspecifically bound [^{125}I]protein A to the filter was washed out as above. Radioimmunolabeled proteins were visualized by autoradiography on radiographic film (Fuji RX).

Isolation of RNA

Total cellular RNA was isolated by guanidinium/cesium chloride method (17) with some modifications. *C. reinhardtii* cells were harvested at the appropriate time by centrifugation and the pellet immediately frozen in liquid nitrogen. The frozen cell pellet was powdered and lysed with 4 M guanidinium isothiocyanate containing 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, and 0.5% (w/v) Sarkosyl NL-97. The lysed cell pellet was dispersed by homogenization and centrifuged for 10 min at 5,000*g*. The supernatant after being drawn into a syringe fitted with 23-gauge needle was layered onto a 4 mL cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) placed in Beckman SW 40 Ultra-clear tube and centrifuged for 15 h at 33,000 rpm (180,000 *g*) and 20°C. The RNA pellet on the bottom of the tube after washing with ethanol was dissolved in 10 mM Tris-HCl (pH 7.5) containing 1% SDS, 5 mM EDTA, and 5% (v/v) phenol. It was then precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol, and stored at −20°C for 2 h. The RNA was recovered by centrifugation and the pellet dissolved in the same solution as above was extracted twice with phenol, once with chloroform, and reprecipitated with ethanol. After the final precipitation, the RNA was washed in 70% ethanol, dissolved in sterile, distilled H_2O, and stored at −80°C.

Northern Hybridization

Equal aliquots of total RNA (10 μg) were denatured and electrophoresed on a formaldehyde containing gel following the method suggested by the manufacturer (Amersham International) with slight modifications. The total RNA in a final volume of 12 μL was mixed with 25 μL formamide, 8 μL formalehyde, and 5 μL 10 × MoPS buffer (pH 7.0) containing 0.2 M MoPS, 0.05 M sodium acetate, and 0.01 M EDTA. After incubating at 65°C for 10 min, the mixture was immediately chilled on ice and 5 μL 50% (v/v) glycerol containing 0.1 mg/mL bromophenol blue was added. The RNA samples were then loaded on 1% (w/v) agarose gel containing 1 × MoPS, 0.07 μg/mL ethidium bromide, and 0.66 M formaldehyde. As a mol wt standard, the RNA Ladder (BRL) was used. The gel was run submerged in 1 × MoPS buffer at 60 V for the first hour and 100 V for the next 3 h. After electrophoresis, the gel was photographed and the RNA capillary blotted onto Zeta-probe membrane (Bio-Rad) overnight with 20 × SSC (3 M NaCl and 0.3 M trisodium citrate) as blotting buffer. The Northern blot after baking at 80°C for 1 h was pre-hybridized and hybridized following the procedure described by Church and Gilbert (5). The probe used to identify CA mRNA in the filter was a terminally ^{32}P-labeled synthetic oligonucleotide whose sequence corresponded to the complementary strand of the CA mRNA sequence. This mRNA sequence (Fig. 1) deduced from the amino acid sequence of *C. reinhardtii* CA containing the zinc-binding sites (12) was designed assuming the biased codon usage found for several nuclear genes (11).

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1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20  21  22  23
Arg Pro Asn Asp Ala Ala Asp Arg Val Thr Ala Val Pro Thr Gln Phe His Phe His Ser Thr Thr Glu
                                                        ===================

3'-GCG GGG TTG CTG CGG CGG CTG GCG CAG TGG CGG CAG GGG TGG GTC AAG GTG AAG GTG AGG TGG TGG CT-5'
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Figure 1. Partial amino acid sequence of *Chlamydomonas* carbonic anhydrase and the corresponding nucleotide sequence of the synthetic oligonucleotide. The region marked by broken lines (= = =) indicates the conserved sequence with animal CAs. The histidine residues are the catalytic zinc-binding sites.

Genomic Southern hybridization experiments have indicated that this probe hybridized with two CA genes in *C. reinhardtii* C-9 cells (S. Fujiwara, H. Fukuzawa, A. Tachiki, S. Miyachi, unpublished data).

RESULTS

Effect of Light on CA Protein Accumulation during Air Adaptation

The time course of CA polypeptide accumulation during the transfer of photoautotrophically grown high-CO_2 cells to low-CO_2 condition was first examined. Soluble protein extracts from cells collected at the different adaptation periods were subjected to SDS-PAGE and the amount of CA protein measured by Western blotting using affinity purified, antideglycosylated CA antibody as a probe. Results in Figure 2 showed an increase in the accumulation of the 37-kD CA monomer 2 h after transfer to low-CO_2 condition. The 37-kD protein is generally a broadband, which on some occasions can be resolved as a doublet signal of approximately 36 to 37 kD as previously reported (3, 6). It is noteworthy that in the Western blots presented here, except for the 37-kD band no other immunosignals were observed indicating the specificity of the affinity-purified antibody against CA.

The effect of light on CA protein accumulation was then examined (Fig. 3). As previously observed, a marked increase in the intensity of 37-kD immunosignal was observed in cells allowed to adapt to air levels of CO_2 for 4 h (lane 3) compared with cells continuously bubbled with 5% CO_2 (lane 1). However, cells transferred to low-CO_2 condition in the dark (lane 2) showed an immunosignal intensity comparable only to high-CO_2 cells indicating that the CA protein did not accumulate in the absence of light. Addition of 10 μM DCMU immediately after transferring the cells from high- to low-CO_2 conditions (lane 4) also caused the same reduction in CA protein accumulation. These results suggest that photosynthesis is needed for the induction of CA protein synthesis in *C. reinhardtii.*

In addition to the photosynthesis dependent process, it was shown previously that a photosynthesis independent blue light process was also involved in the induction of CA activity (9, 10). Western blots of red and/or blue light illuminated air-adapted cell suspension of *C. reinhardtii* revealed that cells illuminated with red light (620–680 nm) alone at an energy fluence rate of 4 W/m^2 at the start of air induction did not show marked accumulation in CA protein after 4 h (lane 1 in Fig. 4A). A marked increase in the intensity of the CA immunosignal, however, was observed when in addition to this red light illumination which is needed for the photosynthesis-dependent process, cells were illuminated at the same time with blue light (460 nm) at an energy fluence rate of 1.3 W/m^2 (lane 2). Similarly, a significant accumulation of CA protein was observed when the cells were illuminated with white light at 6 W/m^2 (lane 3). In the following experiments, only the quality of light was varied with the total energy fluence rate maintained at 5.3 W/m^2 to avoid the possible energy additivity effect attributed to blue light. Results presented in Figure 4B showed that indeed, cells illuminated with blue light compared to those illuminated only with red light exhibited a significant increase in CA protein accumulation.

Effects of Transcription Inhibitors on CA mRNA Accumulation during Air Adaptation

Previous reports have shown that the amount of translatable mRNA for carbonic anhydrase increases upon lowering the CO_2 concentration from 5% to 0.04% (7, 28). However, the

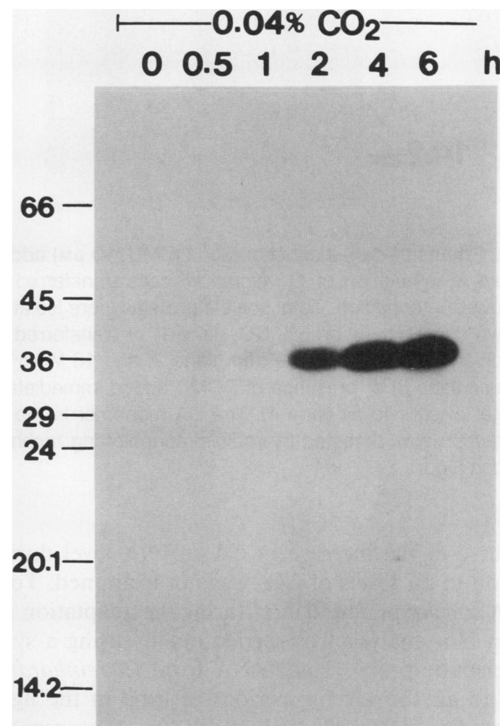

Figure 2. Induction of CA protein accumulation in *C. reinhardtii* during adaptation to air levels of CO_2. High-CO_2 cells were exposed to air levels of CO_2 under continued illumination at 18 W/m^2 and at the indicated time during the air adaptation period cells were harvested and total soluble proteins extracted. The protein extracts were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride filter. The blotted filter was incubated with purified antideglycosylated CA antiserum, and the bound antibody was detected by radioimmunoblot technique as described in "Materials and Methods." Molecular mass markers indicated to the left in the figure are bovine serum albumin (66 kD), ovalbumin (45), glyceraldehyde-3-phosphate dehydrogenase (36), bovine erythrocyte CA (29), trypsinogen (24), trypsin inhibitor (20.1) and α-lactalbumin (14.2).

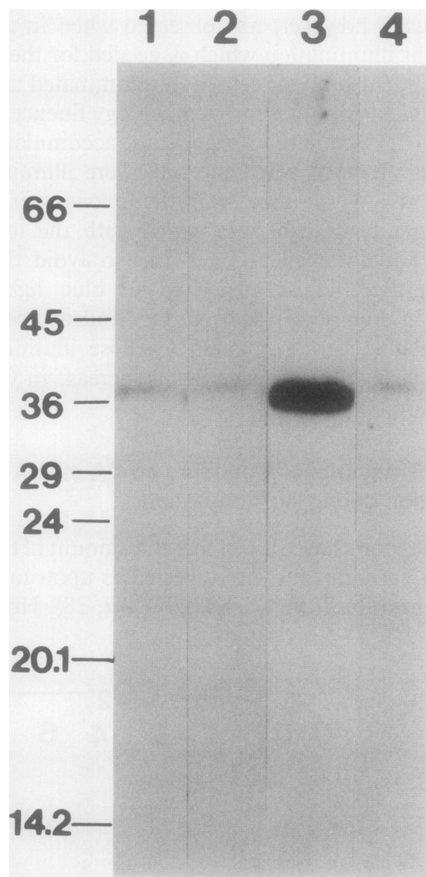

Figure 3. Effects of dark incubation and DCMU (10 μM) addition on CA protein accumulation of *C. reinhardtii* cells transferred from a high- to low-CO_2 condition. Total soluble proteins were isolated from cells grown continuously on 5% CO_2 (lane 1) or transferred to low-CO_2 condition (air) for 4 h in the dark (lane 2) and 18 W/m^2 light in the absence (lane 3) or presence of DCMU added immediately after the transfer of cells to air (lane 4). The CA monomer in the soluble protein fraction was detected by radioimmunoblotting technique as described in Figure 2.

time course of the increase in CA mRNA level during the adaptation to air levels of CO_2 was not examined. To determine CA transcript abundance during the adaptation period, Northern blot analysis was carried out by using a synthetic oligonucleotide probe. Total RNA from *C. reinhardtii* cells exposed to air for varying periods of time in the light was fractionated by electrophoresis on denaturing agarose gel and blotted to Zeta-probe membrane. Figure 5 showed that the ^{32}P-labeled synthetic oligonucleotide probe gave only a single hybridization signal at 2.0-kb mRNA length which is enough to encode the 37-kD CA monomer. This denotes that the 2.0-kb transcript to which the probe specifically hybridized corresponds to CA mRNA. A lag period of 30 min was needed before CA mRNA started to accumulate after the transfer from high- to low-CO_2 condition. After 1-h exposure to air significant amounts of the 2.0-kb CA mRNA were observed with the maximum reached after 2-h exposure, which was then followed by a decline in message abundance.

The effect of two different transcription inhibitors, rifampicin, and actinomycin D, on CA mRNA level during air adaptation was also examined. In *C. reinhardtii* rifampicin has been shown to inhibit chloroplast transcription and rRNA synthesis (27) whereas actinomycin D is a recognized inhibitor of transcription in the cytoplasm. Figure 6A shows the effect of these inhibitors on the level of CA mRNA from cells allowed to adapt to air levels of CO_2 for 4 h. Although rifampicin decreased the accumulation of the chloroplast encoded *rbcL* mRNA (data not shown), it did not, however, affect CA mRNA synthesis. On the other hand, actinomycin D treatment inhibited the accumulation of CA mRNA in air-adapted cells.

The effect of another RNA synthesis inhibitor, 6-methyl purine, as well as the protein synthesis inhibitor, cycloheximide, both used in a previous experiment (9), on CA mRNA level during air adaptation was also examined. Figure 6B shows the effect of these inhibitors on CA mRNA level of cells allowed to adapt to air levels of CO_2 for 4 h. Cells to which 6-methyl purine was added immediately after the transfer to air did not show an accumulation of CA mRNA after

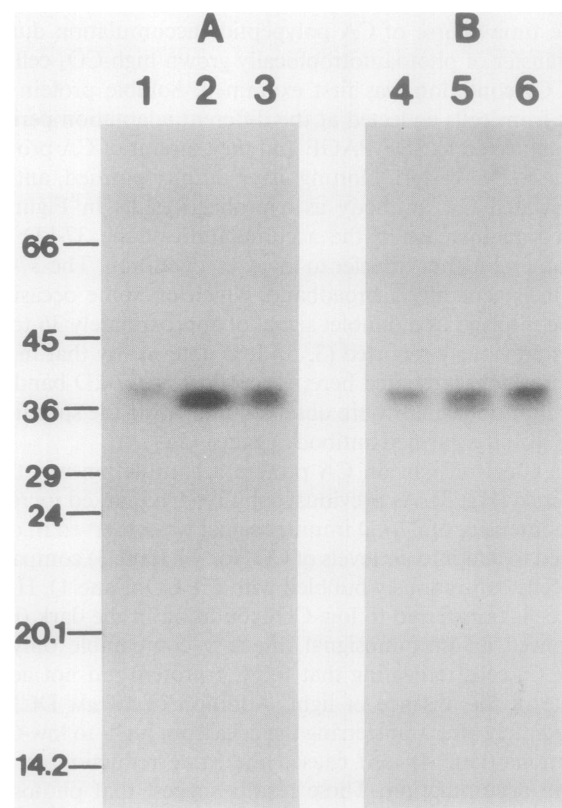

Figure 4. Effects of red and additional blue light illumination on CA protein accumulation of *C. reinhardtii* cells transferred from high- to low-CO_2 condition. A, Upon the transfer to low-CO_2 condition cells were illuminated either with red light (620–680 nm) alone at an intensity of 4 W/m^2 (lane 1) or red light at 4 W/m^2 plus blue light (460 nm) at 1.3 W/m^2 (lane 2) or white light (380–680 nm) at 6 W/m^2 (lane 3). B, Upon transfer to low-CO_2 condition cells were illuminated with red light alone at 5.3 W/m^2 (lane 4) or red light at 4 W/m^2 plus blue light at 1.3 W/m^2 (lane 5) or white light at 5.3 W/m^2 (lane 6). The CA monomer in the soluble protein extracts after the 4 h air adaptation period was detected as in Figure 2.

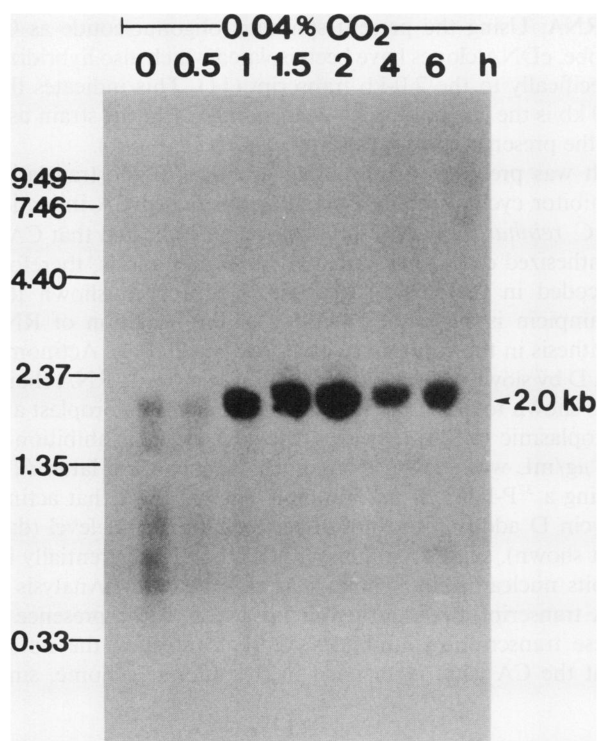

Figure 5. Induction of CA transcript in *C. reinhardtii* during adaptation to air levels of CO_2. High-CO_2 cells illuminated at 18 W/m^2 were bubbled with ordinary air and at the indicated time during the air adaptation period total RNA was isolated. Ten μg RNA was separated on formaldehyde/agarose gel, blotted onto Zeta-probe membrane and hybridized to ^{32}P-labeled synthetic CA oligonucleotide probe as described in "Materials and Methods."

4-h air adaptation (lane 4). Similarly, when 6-methyl purine was added 1 h after the air induction limited accumulation of CA mRNA was observed (lane 5). On the other hand, when cycloheximide was added 1 h after the air induction, accumulation of the CA transcript was observed (lane 6) though comparatively less than the control cells receiving no inhibitor addition (lane 1).

Effect of Light on CA mRNA Accumulation

To determine whether the suppression of CA protein accumulation during exposure to air levels of CO_2 in the dark or in the presence of DCMU was due to a decrease in mRNA abundance, their corresponding mRNA levels were measured by using synthetic oligonucleotide probe. As can be seen in Figure 7, high-CO_2 cells transferred to low-CO_2 condition in the dark (lane 2) did not show significant accumulation of the CA transcript. This is similar to the cells which were air-adapted in the presence of DCMU (lane 4) or the cells maintained at high-CO_2 condition (lane 1).

Similarly, to determine whether the increase in CA protein accumulation in blue light illuminated cells (Fig. 4) was due to an increase in mRNA levels, the CA transcript abundance upon blue light illumination was examined (Fig. 8). Northern hybridization shows a limited accumulation of CA mRNA in cells illuminated with red light alone during the 4-h adaptation period (lane 1). On the other hand, additional illumination with blue light at a low energy fluence rate (panel A) resulted in the induction of the 2.0-kb CA transcript (lane 2) similar to white light irradiated cells (lane 3). When only the quality of light was varied with the total energy fluence rate maintained at 5.3 W/m^2 (panel B), cells illuminated with blue light (lane 5) compared with those illuminated only with red light (lane 4) exhibited a significant increase of transcript accumulation.

DISCUSSION

Through *in vitro* translation experiments it was previously shown that the synthesis of the 37-kD CA peptide was greater with mRNA from low-CO_2 than from high-CO_2 cells, suggesting that CA induction by lowering CO_2 concentration is regulated at transcriptional level (7, 28). Through analysis of the steady-state mRNA levels of high-CO_2 grown cells and air-adapting cells (Figs. 5 and 7) it was confirmed that the adaptation to low-CO_2 condition involves transcriptional reg-

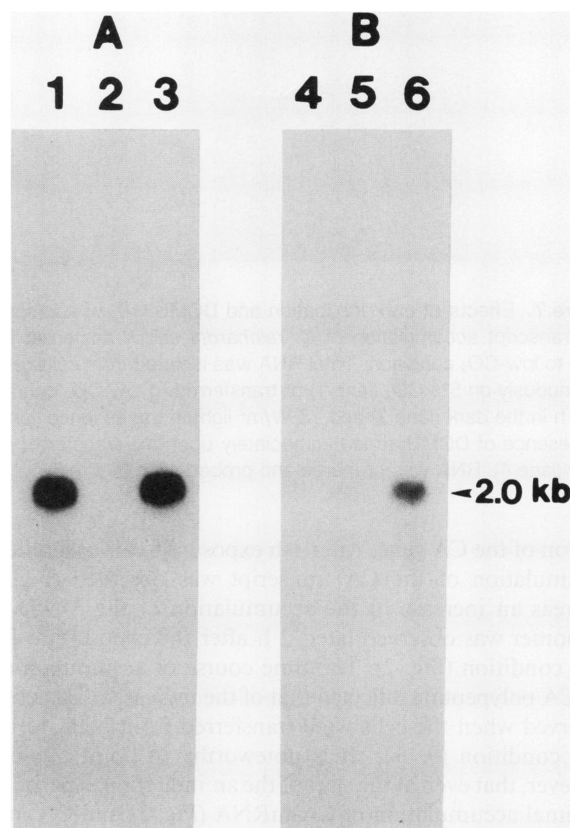

Figure 6. Effects of inhibitors on CA transcript accumulation of air-adapted *C. reinhardtii*. **A,** High-CO_2 cells were exposed to air levels of CO_2 at 18 W/m^2 illumination for 4 h in the absence (lane 1) or presence of 20 μg/mL actinomycin D (lane 2) or 80 μg/mL rifampicin (lane 3) added immediately upon the transfer to low-CO_2 condition. **B,** High-CO_2 cells were exposed to air levels of CO_2 for 4 h in the presence of 2 mM 6-methyl purine (lanes 4 and 5) or 3 μg/mL cycloheximide (lane 6). These inhibitors were added immediately upon the transfer to low-CO_2 condition (lane 4) or after 1 h of air induction (lanes 5 and 6). RNA was prepared and probed as in Figure 5.

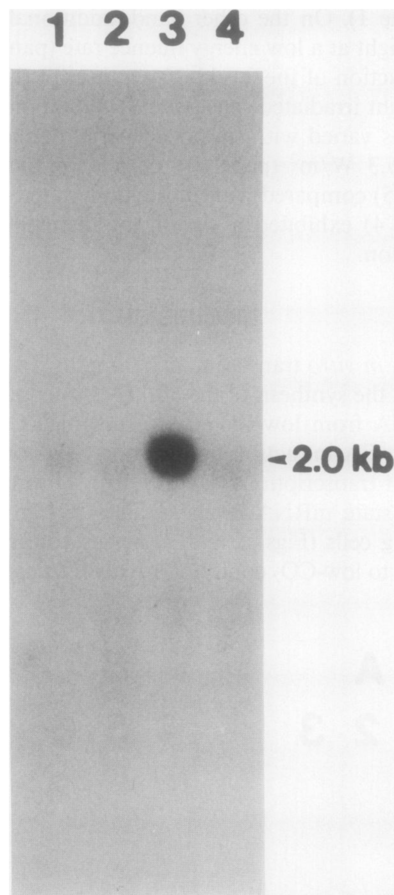

Figure 7. Effects of dark incubation and DCMU (10μM) addition on CA transcript accumulation of *C. reinhardtii* cells transferred from high- to low-CO_2 condition. Total RNA was isolated from cells grown continuously on 5% CO_2 (lane 1) or transferred to low-CO_2 condition for 4 h in the dark (lane 2) and 18 W/m^2 light in the absence (lane 3) or presence of DCMU added immediately upon the transfer of cells to air (lane 4). RNA was prepared and probed as in Figure 5.

ulation of the CA gene. After 1-h exposure to air a significant accumulation of the CA transcript was observed (Fig. 5), whereas an increase in the accumulation of the 37-kD CA monomer was observed later, 2 h after the exposure to low-CO_2 condition (Fig. 2). The time course of accumulation of the CA polypeptide reflected that of the increase in its activity observed when the cells were transferred from high- to low-CO_2 condition (9, 32). It is noteworthy to point out here, however, that even at the start of the air induction experiment, minimal accumulation of CA mRNA (Fig. 5) and CA monomer (Fig. 2) could be observed. This corresponds to the base level of CA activity which can be measured even in high-CO_2 cells (9).

The observed pattern of CA transcript abundance upon exposure to air levels of CO_2 is quite similar to the induction pattern observed by Bailly and Coleman (3) in *C. reinhardtii* 2137 *mt*$^+$ although they reported that mRNA abundance reached its maximum not after 2 but after 3 h exposure to air. However, the length of the CA transcript they reported is shorter (1.4 kb) than the presently observed 2.0-kb CA mRNA. Using the present synthetic oligonucleotide as CA probe, cDNA clones have been isolated which also hybridized specifically to the 2.0-kb transcript (11). This indicates that 2.0 kb is the length of mRNA encoding CA in the strain used in the present experiment (*C. reinhardtii* C-9 *mt*$^-$).

It was previously shown that addition of the translation inhibitor cycloheximide immediately stopped CA induction in *C. reinhardtii* (7, 24, 32). This result indicated that CA is synthesized on cytoplasmic 80*S* ribosomes and is, therefore, encoded in the nuclear genome. It has been shown that rifampicin is a specific inhibitor of the initiation of RNA synthesis in the chloroplast of *C. reinhardtii* (26). Actinomycin D by slowing down the elongation of growing RNA chains was shown to affect the synthesis of both the chloroplast and cytoplasmic rRNA. However, the effectivity of inhibition at 20 μg/mL was shown to be much higher in the latter (27). Using a ^{32}P-labeled *rbcL* probe it was observed that actinomycin D addition did not affect *rbcL* transcript level (data not shown), suggesting that actinomycin D preferentially inhibits nuclear RNA synthesis in *C. reinhardtii*. Analysis of CA transcript levels during air induction in the presence of these transcription inhibitors clearly confirmed the notion that the CA gene is encoded in the nuclear genome, since

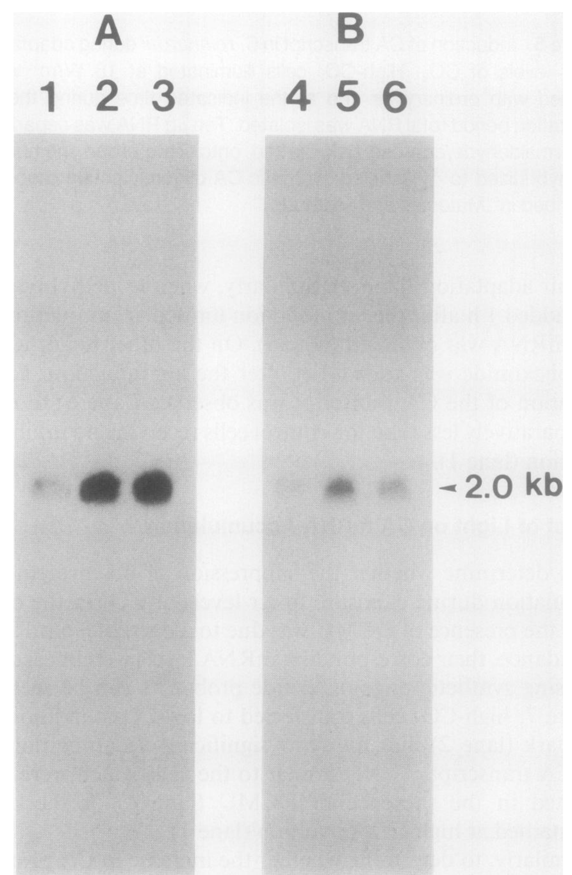

Figure 8. Effects of red and additional blue light illumination on CA transcript accumulation of *C. reinhardtii* cells transferred from high- to low-CO_2 condition. RNA was prepared from the same cultures as described in Figure 4 and probed as indicated in Figure 5.

rifampicin did not affect CA mRNA induction whereas it was greatly inhibited by actinomycin D at 20 μg/mL (Fig. 6A).

Addition of 6-methyl purine at the start of air induction inhibited CA mRNA accumulation (Fig. 6B) similar to the effect of actinomycin D treatment (Fig. 6A). Cycloheximide addition, on the other hand, inhibited translation of CA mRNA while at the same time affecting CA transcript abundance. The observed decrease in the CA mRNA level upon cycloheximide addition might be due to the acceleration of degradation of untranslated transcript or to the inhibition of the synthesis of protein(s) needed for mRNA transcription. It was shown previously that addition of 6-methyl purine 1 h after air induction allowed some increase in CA activity whereas cycloheximide addition resulted in no increase (9). It was further shown that this increase in CA activity after a 1-h air induction could be observed only in the presence of blue light. From these results it was suggested that CA mRNA was formed during the first hour and its translation leading to an increase in CA activity could be observed only in the presence of blue light. Actual measurement of CA transcript abundance confirmed the synthesis of CA mRNA during the first hour (Fig. 5). These results support the previous suggestion that blue light exerts a regulatory effect on the posttranscriptional level of CA biosynthesis. Although most of the reported blue light effect in plants is attributed to transcriptional regulation (14, 21), there is a recent report in *Acetabularia mediterranea* showing that blue light controls UDPG phosphorylase and pyruvate kinase activity at posttranscriptional/translational level (19).

In addition to the posttranscriptional regulation by blue light, present results indicate that blue light is also involved in the induction of CA transcript abundance under low-CO_2 condition (Fig. 8). This blue light induction of CA transcript abundance in turn caused the enhancement of CA protein accumulation (Fig. 4) which parallels the increase in CA activity observed in blue light irradiated cells (10). Blue light illumination at 1.3 W/m^2 in addition to 4 W/m^2 red light illumination caused an increase in CA mRNA abundance compared to cells irradiated only with red light at 5.3 W/m^2 (Fig. 8B), indicating that the enhancement by blue light is not due to the additive effect in terms of energy fluence rate. In fact, as shown previously (10), the enhancing effect of blue light on CA induction was saturated at a low energy fluence rate. However, blue light irradiation alone at 1.3 W/m^2 did not cause an induction of CA transcript abundance (data not shown). Cells had to be simultaneously irradiated with photosynthetic red light in order to induce CA gene expression. From the wavelength dependency curve and KI inhibition experiment (10), it was suggested that flavin is the most likely photoreceptor involved in this blue light induction process.

It was shown previously that CA activity was repressed in air-adapted cells in the dark (9). This can be attributed to the limited accumulation of CA transcripts (Fig. 7) which in turn reflected the limited accumulation of CA protein (Fig. 3). This is in marked contrast to the reported finding by Bailly and Coleman (3) wherein they observed the appearance of the 1.4-kb CA transcript when high-CO_2 cells were transferred to ordinary air in the dark. Although they showed that light, nevertheless, significantly increase CA transcript abundance, they concluded that light is not required for detection of a change in the external DIC concentration. The difference of their results from the present experiment can be attributed to a difference in the strain of *C. reinhardtii* used or a difference in the probe used for Northern hybridization. Toguri *et al.* (30) also suggested that mRNA coding for CA is present in the dark. However, they based this finding not on direct mRNA measurement by Northern hybridization. By pulse-labeling with [^{14}C]arginine followed by immunoprecipitation, they observed the presence of the 42-kD precursor polypeptide during the dark period of the cell cycle and from this suggested that mRNA coding for CA is present in the dark. Also, since they used synchronized *C. reinhardtii* cells grown only in air, it is possible that they were observing CA induction depending on the developmental stage of the cells rather than that due to a lowering of CO_2 concentration as in the case of the present experiment.

Addition of DCMU at the start of air induction inhibited the increase in CA mRNA accumulation similar to the effect observed when the cells were allowed to adapt to air levels of CO_2 in the dark (Fig. 7). This indicates that photosynthesis is essential to the accumulation of CA transcript, thereby confirming the previous suggestion (9) that the photosynthesis-dependent step of CA induction includes the transcriptional process of CA synthesis. This requirement for photosynthesis during mRNA accumulation might indicate that the operation of a complete photosynthetic system is needed for supplying energy or carbon metabolite(s) required for CA induction.

Similar to the present finding that both photosynthesis and blue light regulate CA transcript abundance, some recent reports also suggest the involvement of both photosynthesis and blue light on transcript regulation of other genes. Kindle (14) reported that light caused an increase in *cab*II-1 transcript abundance in *Chlamydomonas* by virtue of its role in the light reactions of photosynthesis and by a blue light stimulated mechanism which is independent of photosynthesis. Roscher and Zetsche (21) showed that in heterotrophically cultured *Chlorogonium elongatum* cells, blue light at low light intensities caused an increase in the amounts of RuBPCase mRNAs. When the cells, however, were shifted to autotrophic condition, the concentration of mRNAs for the large and small subunits of the enzyme was markedly affected by light energy fluence rate, indicative of its dependence on photosynthesis.

The results presented in this paper showed that in addition to low-CO_2 concentration, light is required for the induction of CA transcript abundance in *C. reinhardtii*. Since analysis was made on steady-state CA mRNA levels, delimiting the effect of light and low-CO_2 concentration on CA mRNA transcription or posttranscriptional mRNA processing or CA mRNA degradation could not be done. The point, nevertheless, is that both light and low-CO_2 concentration regulate the CA induction at a level prior to translation. As to the nature of this light requirement, it was established that blue light at a low light intensity is involved in CA transcript regulation together with photosynthesis which is needed to initiate the accumulation of CA transcript.

Since two genes encoding periplasmic CA polypeptides have been identified (S. Fujiwara, H. Fukuzawa, A. Tachiki, S. Miyachi, unpublished data) using the oligonucleotide

probe, which was used to monitor the CA mRNA levels in this study, it is needed to clarify how each gene is controlled by DIC concentration and light.

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