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CO_2-Responsive Transcriptional Regulation of *CAH1* Encoding Carbonic Anhydrase Is Mediated by Enhancer and Silencer Regions in *Chlamydomonas reinhardtii*[1]

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Chlamydomonas reinhardtii **adapts to the stress of CO_2-limiting conditions through the induction of a set of genes including *CAH1*, which encodes a periplasmic carbonic anhydrase. *CAH1* is up-regulated under low-CO_2 conditions (air containing 0.04% [v/v] CO_2) in the presence of light, whereas it is down-regulated under high-CO_2 conditions (5% [v/v] CO_2) or in the dark. In an effort to identify cis-elements involved in the transcriptional regulation of *CAH1*, a series of 5′-nested deletions of the region upstream of *CAH1* were fused to a promoterless arylsulfatase reporter gene (*ARS*). The upstream region from −651 to +41 relative to the transcription start site was sufficient to regulate the expression of *ARS* with kinetics similar to those of endogenous *CAH1*. Deletion of the region between −651 and −294 resulted in a significant decrease in the level of arylsulfatase activity expressed under low-CO_2 conditions. The 543-bp upstream region from −651 to −109, without any promoter elements, CAAT-box, or TATA-box, could confer CO_2 and light responsiveness on the β_2-tubulin minimal promoter. This 543-bp region was divided into two parts: a 358-bp silencer region from −651 to −294, which represses the minimal promoter activity under high-CO_2 conditions, and a 185-bp enhancer region from −293 to −109, which activates the promoter under low-CO_2 conditions in the presence of light.**

When microalgae are exposed to the stress of CO_2-limiting conditions, they express a set of genes involved in a carbon-concentrating mechanism (CCM) that functions to take up inorganic carbon from the external environment into the cells and to elevate the CO_2 level around Rubisco (Aizawa and Miyachi, 1986; Badger and Price, 1994). This adaptation to the stress of low-CO_2 conditions suggests the existence of a sensory mechanism by which cells perceive changes in the levels of environmental CO_2 and a pathway by which the signal indicative of the shortage of CO_2 is transduced into the induction of specific genes.

In *Chlamydomonas reinhardtii*, several genes regulated in response to changes in CO_2 concentration have been isolated, including Ala:α-ketoglutarate aminotransferase (Chen et al., 1996), mitochondrial CA (Eriksson et al., 1996), and chloroplast envelope protein LIP-36 (Chen et al., 1997). *CAH1*, encoding a periplasmic CA, is one of the most well characterized of these genes (Dionisio-Sese et al., 1990; Fujiwara et al., 1990; Fukuzawa et al., 1990). *CAH1* is not transcribed when cells are maintained under high-CO_2 conditions, whereas transcripts of this gene accumulate at a significant level within 1 h after transfer to low-CO_2 conditions. However, this induction does not occur when cells are cultured in the dark or in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, indicating that photosynthetic electron flow is required. Interestingly, *CAH2* is regulated in a manner opposite to that of *CAH1*. It is transcribed preferentially under high-CO_2 conditions, even in the dark, and light has a negative effect on the expression of *CAH2* (Fujiwara et al., 1990).

Ronen-Tarazi et al. (1995) have described an analysis of a low-CO_2-inducible promoter in a cyanobacterium, *Synechococcus* sp. PCC7942, produced by fusing a 380-bp fragment derived from the region upstream of *cmpA* with a promoterless chloramphenicol acetyltransferase reporter gene. However, no detailed analysis of the CO_2-responsive elements in this strain has been reported.

In eukaryotes, an analysis of CO_2-responsive promoters of *β-CA1* and *β-CA2*, which encode mitochondrial CA isozymes in *C. reinhardtii*, has been reported (Villand et al., 1997). Both of these genes are up-regulated under low-CO_2 conditions and they share an identical 194-bp sequence in their 5′-upstream region. This 194-bp region was shown to be enough to drive a promoterless *ARS* reporter gene. However, it is not known whether this CO_2-dependent gene regulation is mediated by activation under low-CO_2 conditions or by repression under high-CO_2 conditions.

In this paper, we present direct evidence, from experiments using a series of chimeric fusion constructs containing *CAH1* upstream regions, the β_2-tubulin minimal promoter and an *ARS* reporter gene, indicating that the CO_2-responsive transcriptional regulation of *CAH1* is mediated by both enhancer and silencer regions in its 5′-upstream region.

MATERIALS AND METHODS

Cells and Culture Conditions

Chlamydomonas reinhardtii strain 5D (*nit1-305, cw-15*) (Tam and Lefebvre, 1993) was maintained in TAP medium

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(Gorman and Levine, 1965) under continuous illumination (150 μmol m^{-2} s^{-1}) at 28°C. For the high-CO_2 conditions, cells were cultured in modified HSM medium (Sueoka, 1960), supplemented with 20 mM MOPS (pH 7.2) and 0.4 mM $MgSO_4$ (HSM+S) under aeration with air enriched with 5% (v/v) CO_2. For low-CO_2 conditions, cultures were bubbled with ordinary air containing 0.04% (v/v) CO_2.

Chimeric Constructs

DNA fragments of various lengths containing portions of the 5′-upstream region of *CAH1* were amplified by PCR. The oligonucleotide primer p-Cla1 (5′-GCATCGGTGTTCA AGTGGGTTGCAGGTA-3′), which is complementary to the *CAH1* upstream sequence from position +20 to +41 relative to the transcription initiation site, was used in combination with p-Xho2 (5′-TTCTCGAGGTTCTCCACCTTGCCAGCG CAC-3′), p-Xho3 (5′-TTCTCGAGTCAGCTTCTCTCCCGC CAGCAT-3′), p-Xho4 (5′-ATCTCGAGAGATTTTCACCG GTTGGAAGGA-3′), p-Xho5 (5′-AACTCGAGGTATGAC ATGGGTGCCGGAACT-3′) and p-Xho6 (5′-CCCTCGAG GCTGCAGACTGTGCGCATGCAG-3′), to amplify upstream regions between −818 and +41, −651 and +41, −293 and +41, −200 and +41, and −151 and +41, respectively. These PCR products were blunt-ended, phosphorylated, *Xho*I- digested, and inserted into the *Sal*I-*Eco*RV restriction sites of the plasmid pJD54, which contains a promoterless *ARS* gene (Davies et al., 1992). These chimeric constructs were named pCAO2, pCAO3, pCAO4, pCAO5, and pCAO6, respectively.

To generate constructs containing the β_2-tubulin minimal promoter (pCT series), two additional primers were synthesized. CAup-Kpn4 (5′-ATGGTACCTTAAAACCAGAAGCT GCATTTC-3′) hybridizes to the region just upstream of the CAAT box (between −109 and −130) and CAup-Kpn7 (5′-ATCAGCTGACAACGCTGCCAACGTGGTGGC-3′) corresponds to the region between −294 and −315. Primer sets p-Xho3 and CAup-Kpn4, p-Xho3 and CAup-Kpn7, and p-Xho4 and CAup-Kpn4 were used for PCR. The amplified fragments were cloned into the blunt-ended *Kpn*I site of the plasmid pJD100 (Davies and Grossman, 1994), and the resulting chimeric constructs were named pCT34, pCT37, and pCT44, respectively.

Transformation

The host *Chlamydomonas* strain 5D (*nit1-305, cw-15*) was co-transformed with the chimeric constructs and plasmid pMN24, containing the entire nitrate reductase gene (Fernandez et al., 1989), by the glass beads method with slight modifications (Kindle, 1990). Cells cultured to a concentration of 1 to 2 × 10^6 cells mL^{-1} were collected by centrifugation, and resuspended in TAP(NO_3) (TAP medium in which NH_4Cl was replaced by KNO_3) at a concentration of 2 × 10^8 cells mL^{-1}. Ten micrograms of pMN24 and 50 μg of a chimeric construct were added to 5 mL of the cell suspension as supercoiled DNA, and vortexed with glass beads for 30 s. The glass beads were allowed to settle, and the supernatant was diluted with TAP(NO_3) medium and centrifuged. The pelleted cells were resuspended in TAP(NO_3) and spread onto TAP(NO_3) agar plates. After 1 week, the *nit*$^+$ colonies that appeared on the plates were used for further analysis.

Screening of *ARS*-Expressing Transformants

The cells from *nit*$^+$ colonies were grown in liquid TAP(NO_3) medium in 96-well microtiter plates. These cultures were used to inoculate HSM+S medium containing 0.3 mM X-SO_4 (Sigma, St. Louis). The cells were cultured under high-CO_2 conditions for 1 d, and then transferred to low-CO_2 conditions. Transformants expressing arylsulfatase were identified as those showing a blue color. As a more sensitive assay, 0.8 mM N-SO_4 (Sigma) was used as a substrate. To the culture, an equal volume of a solution containing 4% (w/v) SDS and 0.4 M Na acetate (pH 4.8) and 0.2 volume of 10 mg mL^{-1} tetrazotized-*o*-dianisidine (Sigma) were added to visualize the arylsulfatase activity.

Quantification of Arylsulfatase Activity

Cell cultures were centrifuged and the supernatants were assayed for arylsulfatase activity by adding 50 μL of 100 mM imidazole, 25 μL of 1 M Tris-HCl (pH 10.0), and 5 μL of 80 mM N-SO_4 to 420 μL of supernatant (Ohresser et al., 1997). The mixture was incubated at 37°C, and the reaction was stopped by adding 500 μL of a solution containing 4% (w/v) SDS and 0.4 M Na acetate (pH 4.8). The absorbance was measured at 540 nm immediately after addition of 100 μL of 10 mg mL^{-1} tetrazotized-*o*-dianisidine, and the value was normalized by dividing by the chlorophyll content of the culture.

Northern-Blot Analysis

Total RNA was isolated as described by Chomczynski et al. (1987). Ten micrograms of total RNA was electrophoresed in a denaturing agarose gel and blotted onto a nylon membrane (Hybond-N+, Amersham). Two radiolabeled probes were used for hybridization, a ^{32}P-terminally labeled 40-mer oligonucleotide (p-CA1-5′; 5′-GGTGT-TCAAGTGGGTTGCAGGTAATGACTCAACGCAGGGT-3′), which hybridizes to the 5′ untranslated region of *CAH1*, and 0.8- and 1.2-kb *Bam*HI fragments corresponding to the *ARS* coding region, which were excised from plasmid pJD27 containing *ARS* cDNA (de Hostos et al., 1989).

RESULTS

A 692-bp Region between −651 and +41 Sufficient for CO_2- and Light-Responsive Gene Regulation

In an effort to identify the regions involved in CO_2-responsive transcriptional regulation of *CAH1*, a series of 5′-nested deletions produced by PCR were fused to an *ARS* reporter gene (Davies et al., 1992). Five chimeric constructs were generated, named pCAO2, pCAO3, pCAO4, pCAO5, and pCAO6, that contain portions of the upstream region up to positions −818, −651, −293, −200, and −151, respectively (Fig. 1). These constructs were introduced into the

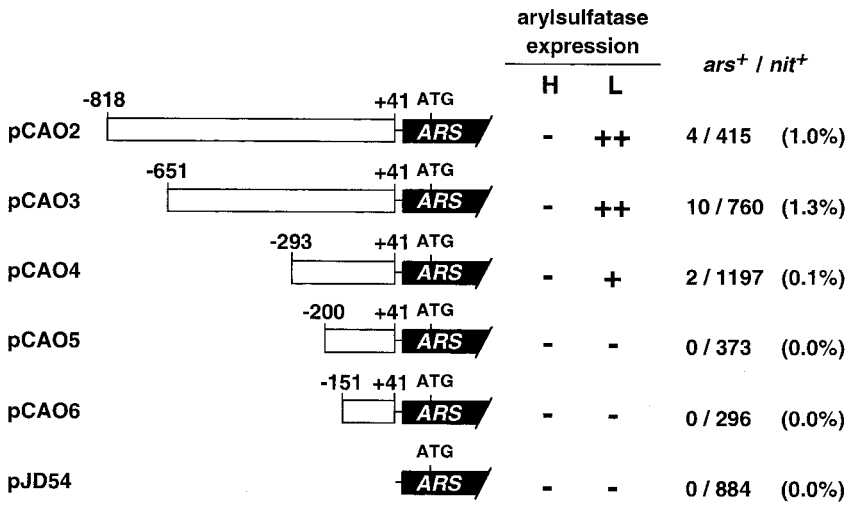

Figure 1. Schematic drawings of the chimeric constructs. A series of 5′-nested deletions of the *CAH1* upstream region, represented by white boxes, were fused to the promoterless *ARS* reporter gene, represented by black bars. Numbering on the white boxes indicates positions relative to the transcription start site. Strain 5D was co-transformed with these chimeric constructs and pMN24, and *nit*$^+$ transformants were analyzed for arylsulfatase expression under high- (H) and low-CO_2 conditions (L). High (++), low (+), or no (−) arylsulfatase activity is indicated. The number of arylsulfatase-expressing colonies among the *nit*$^+$ transformants is indicated at the far right as *ars*$^+$/*nit*$^+$. The nucleotide sequence of the 5′-upstream region of *CAH1* will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession no. AB026126.

host *Chlamydomonas* strain 5D (*nit1-305, cw-15*) together with pMN24 DNA (Fernandez et al., 1989), and *nit*$^+$ colonies exhibiting arylsulfatase activity under low-CO_2 conditions were selected. When cells were transformed with pCAO2 or pCAO3, 1.0% and 1.3% of the total *nit*$^+$ colonies exhibited arylsulfatase activity. However, when cells were transformed with pCAO4 only 0.1% of the *nit*$^+$ colonies expressed arylsulfatase activity, and the levels of activity were much lower than those obtained with pCAO2 or pCAO3. When cells were transformed with pCAO5 or pCAO6, no transformants expressing arylsulfatase activity were obtained among the *nit*$^+$ colonies tested (Fig. 1).

Among the arylsulfatase-expressing clones, transformants containing the appropriate *CAH1* upstream regions, which had been generated by PCR, were selected by Southern-blot analysis (data not shown). The arylsulfatase activity expressed by the individual transformants was quantified under high-CO_2 conditions in the light, under low-CO_2 conditions in the light, and under low-CO_2 conditions in the dark (Fig. 2). After 8 h of incubation under these conditions, arylsulfatase activity was measured by a 12-h enzyme reaction as described in "Materials and Methods." No activity was detected in the host strain 5D because the endogenous *ARS* gene is repressed by excess SO_4^{2-} in the culture medium (de Hostos et al., 1988). The strains CAO2 and CAO3, harboring pCAO2 and pCAO3, respectively, exhibited arylsulfatase activity only under low-CO_2 conditions in the light, indicating that the 692-bp region between −651 and +41 was sufficient for CO_2-responsive regulation. Strain CAO4 harboring pCAO4 also exhibited arylsulfatase activity under low-CO_2 conditions in the light, but the level of activity was much lower than that of CAO2 or CAO3. This result suggests that the 358-bp region between −651 and −294 may function as an enhancer element under low-CO_2 conditions.

The expression of *CAH1* is not induced in the dark, even if cells are shifted from high- to low-CO_2 conditions (Fujiwara et al., 1990; Fukuzawa et al., 1990). The light-requirement for arylsulfatase induction in the transformants was also tested as shown in Figure 2. Light-dependent expression of arylsulfatase activity was observed in all of

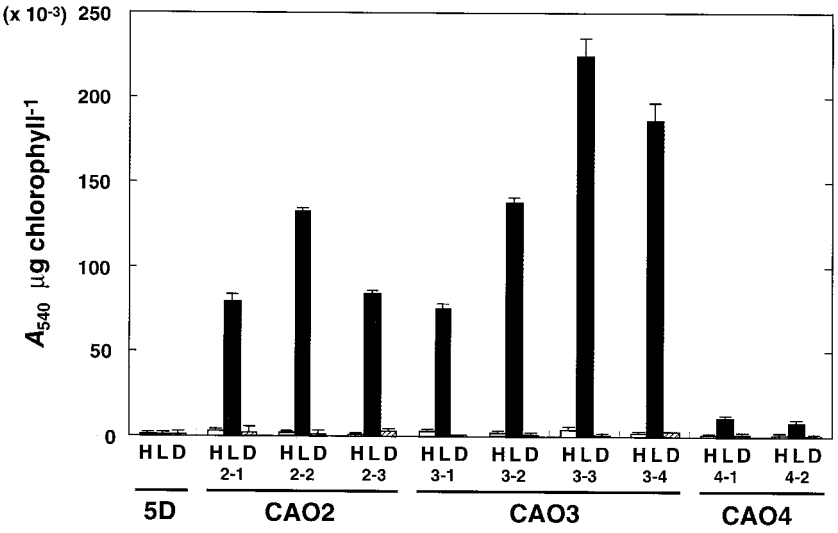

Figure 2. Quantification of arylsulfatase activity in individual transformants. Cells cultured under high-CO_2 conditions were maintained under high-CO_2 in the light (H), transferred to low-CO_2 in the light (L), or to low-CO_2 in the dark (D). After an 8-h incubation under these conditions, arylsulfatase activity was measured by a 12-h enzyme reaction, as described in "Materials and Methods." 5D is the host strain used for DNA transformation. CAO2, CAO3, and CAO4 represent transformants harboring pCAO2, pCAO3, and pCAO4, respectively. The results are the average of three determinations, with SD represented by the bars above the graph.

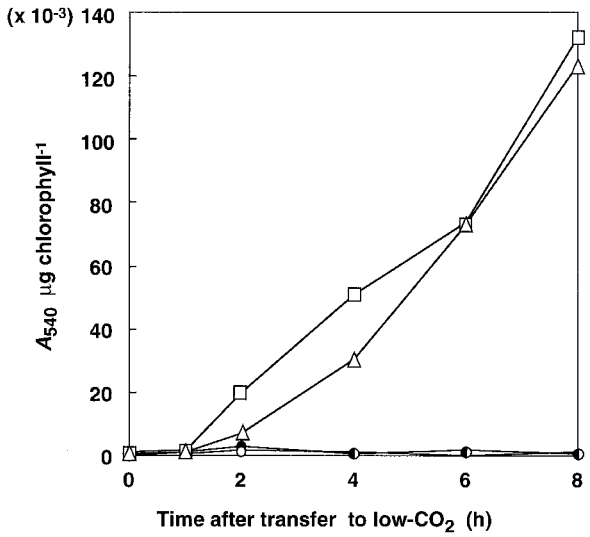

Figure 3. Time course of changes in arylsulfatase activity in transformants harboring the chimeric constructs. Cells grown under high-CO_2 conditions were transferred to low-CO_2 conditions or maintained under high-CO_2 conditions. The arylsulfatase activity was measured by 3-h enzyme reactions as described in "Materials and Methods." □, CAO2–1, low-CO_2; △, CAO3–1, low-CO_2; ●, 5D, high-CO_2; ○, 5D, low-CO_2.

the tested transformants containing the chimeric constructs pCAO2, pCAO3, and pCAO4.

The arylsulfatase activity in strains CAO2-1 and CAO3-1 was measured at various times after transfer from high- to low-CO_2 conditions by 3-h enzyme reactions as described in "Materials and Methods" (Fig. 3). Under high-CO_2 conditions, the activity in both strains remained undetectable as in the host strain 5D (data not shown). Activity was detected 2 h after transfer to low-CO_2 conditions and the levels increased for up to 8 h. This induction of arylsulfatase activity showed a good correlation with the induction of periplasmic CA, which accumulates within 2 h after transfer to low-CO_2 conditions and continues to increase for up to 8 h (Dionisio-Sese et al., 1990; Rawat and Moroney, 1995). These results suggest that the 692-bp region between −651 and +41 is sufficient to regulate the expression of the *ARS* reporter gene with kinetics similar to those of endogenous *CAH1*.

Accumulation of *CAH1/ARS* Chimeric mRNA in Transgenic *Chlamydomonas* Cells

To evaluate the expression of the *ARS* reporter gene at the mRNA level, 10 μg of total RNA from each of the transgenic *Chlamydomonas* strains CAO2-1 and CAO3-1, isolated 0, 1, 2, 4, and 6 h after transfer from high- to low-CO_2 conditions, was electrophoresed and hybridized with a ^{32}P-labeled oligonucleotide probe, p-CA1-5′, which is specific for the 5′-untranslated region of *CAH1* (Fig. 4A). This probe hybridized with both transcripts from the endogenous *CAH1* and those from the introduced chimeric constructs containing the 5′-flank of *CAH1* and the *ARS* coding region. In all strains tested, 2.0-kb *CAH1* mRNA was detected 1 h after transfer to low-CO_2 conditions, and the amount reached a maximum 4 h after the transfer. In CAO2-1 and CAO3-1, 2.5-kb transcripts from the chimeric construct (*CAH1/ARS*) were expressed, showing kinetics similar to that of endogenous *CAH1*. To distinguish the *CAH1/ARS* chimeric transcripts from the premature *CAH1* mRNA observed in lanes containing RNA from the host strain 5D, the membrane was reprobed with ^{32}P-labeled *ARS* cDNA (Fig. 4B). The chimeric transcripts accumulated only in the transgenic *Chlamydomonas* cells grown under low-CO_2 conditions, and no signal was detected in the case of the host strain 5D under either high- or low-CO_2 conditions. These results indicate that CO_2-responsive expression of arylsulfatase encoded by pCAO2 and pCAO3 was regulated at the mRNA level with kinetics similar to endogenous *CAH1*.

To examine the effect of light on induction of the *CAH1/ARS* chimeric genes in CAO2-1 and CAO3-1, total RNA samples were isolated from cells grown under low-CO_2 conditions in the dark or in the light for 4 h, and hybridization was performed using ^{32}P-labeled p-CA1-5′ as a

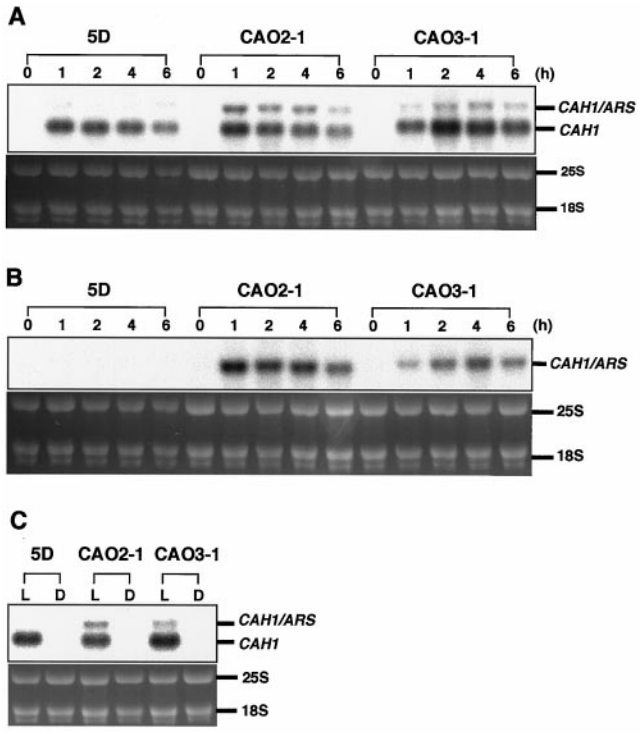

Figure 4. Northern-blot analyses of *CAH1/ARS* chimeric transcripts in the transformants CAO2-1 and CAO3-1. A and B, Cells grown under high-CO_2 conditions were transferred to low-CO_2 conditions and total RNA was isolated 0, 1, 2, 4, and 6 h after the change in CO_2 level. Ten micrograms of RNA from each of the transformants was electrophoresed in a denaturing agarose gel, blotted onto a membrane, and hybridized with ^{32}P-labeled p-CA1-5′ (A) or *ARS* cDNA (B). *CAH1/ARS* represents the 2.5-kb transcripts from the chimeric constructs. C, CAO2-1 and CAO3-1 were grown under high-CO_2 conditions in the light and subsequently transferred to low-CO_2 conditions in the light (L) or dark (D). Total RNA was isolated 4 h after transfer to each of these conditions. The oligonucleotide probe p-CA1-5′ was used for hybridization.

probe (Fig. 4C). Chimeric transcripts or transcripts from the endogenous *CAH1* did not accumulate in the dark, indicating that the *CAH1/ARS* chimeric genes in pCAO2 and pCAO3 are regulated by light at the mRNA level.

A 543-bp Region between −651 and −109 Conferring CO_2 Responsiveness on the β_2-Tubulin Minimal Promoter

The region between −651 and +41 contains the transcriptional start site and putative promoter elements, TATA- and CAAT boxes. To test whether CO_2-responsive regulatory cis-elements are located in the region upstream of the promoter region, the 543-bp region between -651 and -109, which does not contain these promoter elements, was inserted into the plasmid pJD100 (Davies and Grossman, 1994). In pJD100, the *ARS* reporter gene is driven by a constitutive β_2-tubulin minimal promoter. The resulting plasmid, pCT34 (Fig. 5A) was introduced into *Chlamydomonas* cells and transformants harboring only one copy of the intact chimeric construct were grown under high- and low-CO_2 conditions for 4 h. Arylsulfatase activity and mRNA levels were measured (Fig. 5, B and C). Transformants harboring the chimeric construct pJD100 or pCT34 were named T strains and CT34 strains, respectively. It was reported that pJD100 drives low-level constitutive arylsulfatase expression when transformants are grown heterotrophically in TAP medium (Davies and Grossman, 1994). However, under photoautotrophic conditions (Fig. 5B), the T strains exhibited slightly higher levels of arylsulfatase activity under high-CO_2 conditions than low-CO_2 conditions. Two representative graphs of the activity displayed by six T strains tested are shown in Figure 5B. In *C. reinhardtii*, tubulin genes are up-regulated during cell division for the formation of the mitotic spindle apparatus and for the assembly of new flagella (Ares and Howell, 1982), and expression of pJD100 is reported to increase slightly during cell division (Davies and Grossman, 1994). It is supposed that higher levels of expression of pJD100 were observed in T strains under high-CO_2 conditions because the cells divided more frequently under high- than under low-CO_2 conditions.

On the other hand, transformants CT34-1 and CT34-2 expressed arylsulfatase activity with the same kinetics as endogenous *CAH1* (Fig. 5B). Under high-CO_2 conditions, they exhibited reduced activity compared with T-1 and T-2. Northern-blot analysis revealed that the reduction in arylsulfatase activity was due to a decrease in the levels of chimeric transcripts expressed from the β_2-tubulin-*ARS* hybrid gene (β_2-*TUB/ARS*) (Fig. 5C). These results indicate that the 543-bp region functions as a transcriptional silencer under high-CO_2 conditions.

Under low-CO_2 conditions, these transformants exhibited 3- to 7-fold higher levels of activity and accumulated 6- to 20-fold higher levels of the β_2-*TUB/ARS* mRNA compared with T strains harboring the β_2-tubulin minimal promoter-driven *ARS*. These results indicate that the 543-bp region also functions as a transcriptional enhancer under low-CO_2 conditions. An additional band observed above the β_2-*TUB/ARS* mRNA (Fig. 5C) may be premature mRNA containing *ARS* introns.

The 543-bp Region Is Divided into a Silencer Region and an Enhancer Region

To identify which parts of the 543-bp region function as a silencer or an enhancer, the 543-bp fragment was divided into two parts: a 358-bp region from −651 to −294, and a 185-bp region from −293 to −109. The two fragments were

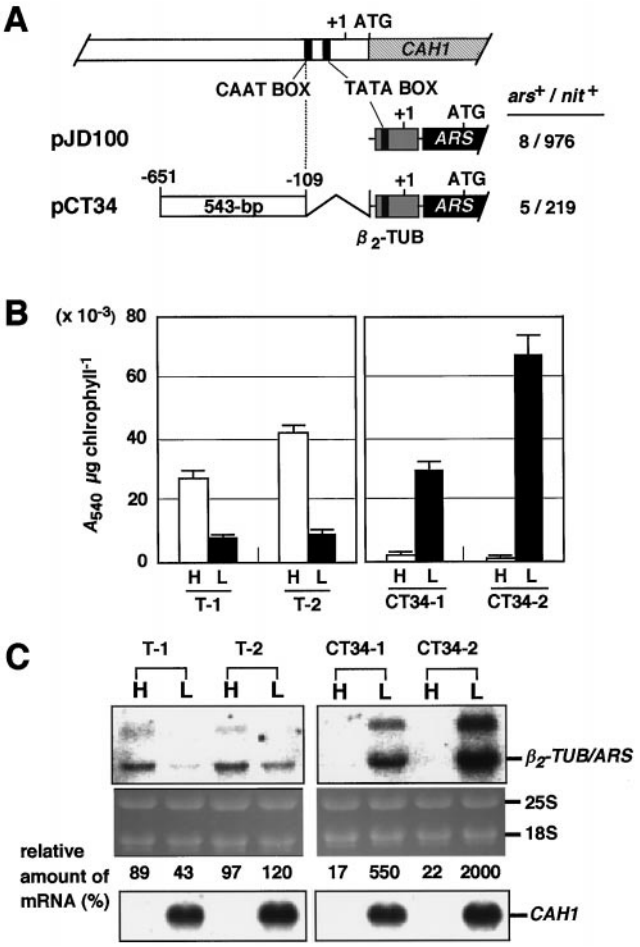

Figure 5. A, Schematic drawings of the chimeric constructs. The 543-bp region from −651 to −109 (white box), which does not include sequences downstream from the CAAT box, was fused to a β_2-tubulin minimal promoter (shaded box)-driven *ARS* reporter gene (pJD100), and the resulting chimeric construct was named pCT34. B, Quantification of arylsulfatase activity in individual transformants. The activity in at least five independent transformants was measured, and the results for two representative transformants are shown. Cells grown under high-CO_2 conditions were maintained under high-CO_2 (H) or transferred to low-CO_2 conditions (L), and the arylsulfatase activity was measured 4 h after the change in CO_2 level. Bars above the graph indicate the SD for the average of three determinations. C, Northern-blot analysis of the β_2-*TUB/ARS* chimeric transcripts in transformants T-1, T-2, CT34-1, and CT34-2. Total RNA was isolated from cells adapted to high- (H) or low-CO_2 conditions (L) for 4 h. Ten micrograms of RNA was electrophoresed and hybridized with ^{32}P-labeled *ARS* cDNA (upper autoradiographs) or p-CA1-5′ (lower autoradiographs). β_2-*TUB/ARS* represents the transcript from the chimeric constructs. Values of the relative amount of mRNA represent the percentage of the amount of the chimeric transcript over the average amount in three independent T strains (T-1, T-2, and T-3; not shown).

then inserted into pJD100 to generate pCT37 and pCT44 (Fig. 6A). Transformants harboring the chimeric construct pCT37 or pCT44 were named CT37 and CT44, respectively.

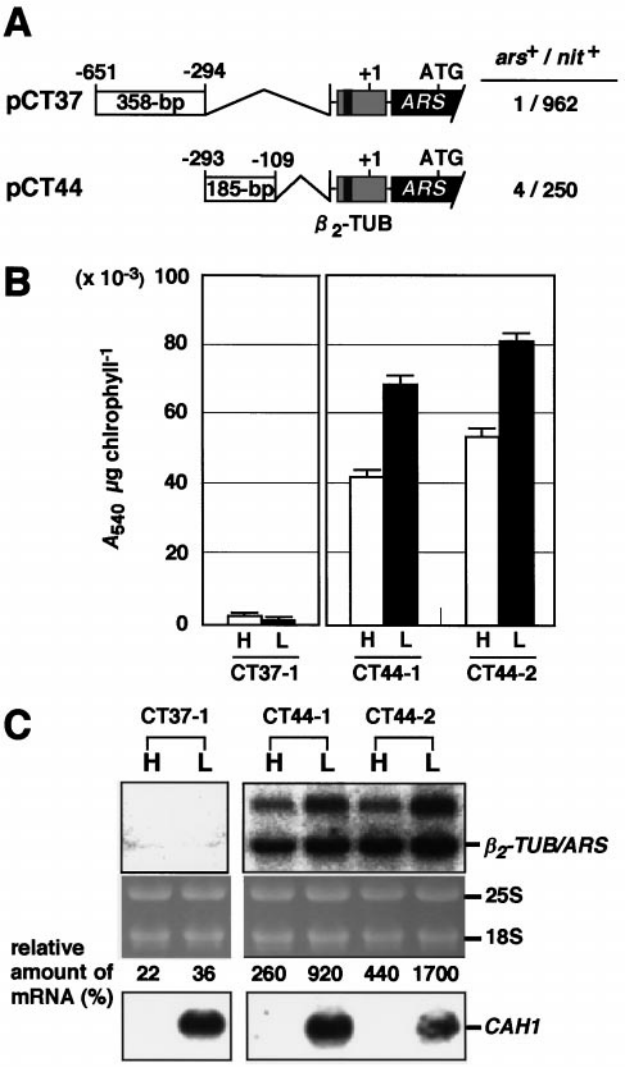

Figure 6. A, Schematic drawings of the chimeric constructs. The *CAH1* 5′-upstream region between −651 and −109 was divided into two fragments at position −293 and each fragment was fused to the *ARS* reporter gene driven by the β_2-tubulin minimal promoter. The chimeric construct pCT37 contains the 385-bp region from −651 to −294, and pCT44 contains the 185-bp region from −293 to −109, respectively. B, Quantification of arylsulfatase activity in individual transformants. The arylsulfatase activity under high- (H) and low-CO_2 conditions (L) was quantified in transformants containing pCT37 (CT37-1) or pCT44 (CT44-1 and CT44-2). Results for two representative CT44 strains among four tested are shown. Bars above the graph indicate the SD for the average of three determinations. C, Northern-blot analysis of β_2-*TUB/ARS* chimeric transcripts in transformants CT37, CT44-1, and CT44-2. Ten micrograms of total RNA from cells exposed to high- (H) or low-CO_2 conditions (L) for 4 h was electrophoresed in each lane. Radiolabeled *ARS* cDNA (upper autoradiographs) or p-CA1-5′ (lower autoradiographs) were used as probes. Values of the relative amount of mRNA represent the percentage of the amount of the chimeric transcripts in CT37 and CT44 over the average amount in three independent T strains.

It was expected that the 358-bp region functioned as an low-CO_2-dependent enhancer, because deletion of this region from pCAO3 resulted in a decrease in the level of arylsulfatase activity under low-CO_2 conditions (Figs. 1 and 2). Unexpectedly, however, in the presence of this region, the basal activity of the β_2-tubulin minimal promoter was repressed under both high- and low-CO_2 conditions (CT37-1 in Fig. 6B). Northern-blot analysis revealed that the amount of β_2-*TUB/ARS* mRNA in CT37-1 was less than one-third of that in T strains (Fig. 6C). These results suggest that the 358-bp region functions as a transcriptional silencer independent of the external CO_2 level.

In the transformants CT44-1 and CT44-2 harboring pCT44 (Fig. 6A), which does not contain the 358-bp region, arylsulfatase expression was no longer repressed under high-CO_2 conditions. The arylsulfatase activity levels in two representatives of four strains tested are shown in Figure 6B (CT44-1 and CT44-2). This result strongly supports our speculation that the region from -651 to -294 functions as a silencer. Furthermore, under low-CO_2 conditions, the CT44 strains (CT44-1 and CT44-2) exhibited 8- to 10-fold higher arylsulfatase activity (Fig. 6B) and accumulated 9- to 17-fold higher levels of β_2-*TUB/ARS* mRNA (Fig. 6C) than those in T-1 and T-2 cells containing pJD100 (Fig. 5, B and C), indicating that the 185-bp region functions as a transcriptional enhancer under low-CO_2 conditions. These results indicate that both the 358-bp silencer region from −651 to −294 and the 185-bp enhancer region from −293 to −109 are sufficient to regulate the expression of *CAH1* in response to the external CO_2 level.

The light responsiveness of these chimeric constructs was also assayed by northern-blot analysis using total RNA samples isolated from cells incubated under high- or low-CO_2 conditions in the light or in the dark. In CT34-2, the chimeric transcript accumulated under low-CO_2 conditions only in the light as in the case of the endogenous *CAH1* (Fig. 7). This result suggests that the 543-bp region is sufficient for light-dependent transcriptional regulation.

In the presence of the 185-bp region, 2-fold higher levels of β_2-*TUB/ARS* mRNA were expressed even in the dark when the cells were exposed to low-CO_2 conditions (CT44-2 in Fig. 7) compared with T-2 in the dark. When CT44-2 cells were cultured under low-CO_2 conditions in the light, a 12-fold higher level of β_2-*TUB/ARS* mRNA was detected compared with that in T-2 cells. These findings suggest that the 185-bp region is sufficient to confer light-dependent responsiveness on the β_2-tubulin minimal promoter.

To clarify whether the repression under high-CO_2 conditions is dependent on light, expression of the chimeric constructs was examined under high-CO_2 conditions both in the light and in the dark (Fig. 7). In the presence of the 358-bp region (CT34-2 and CT37-1 in Fig. 7), β_2-*TUB/ARS* mRNA was scarcely detected under high-CO_2 conditions regardless of illumination. In CT44-2 harboring pCT44, which lacks the 358-bp region, β_2-*TUB/ARS* mRNA accumulated under high-CO_2 conditions both in the light and in the dark. These results indicate that the silencer element represses transcription in a manner independent of exposure to light. Furthermore, when CT37-1 cells were cul-

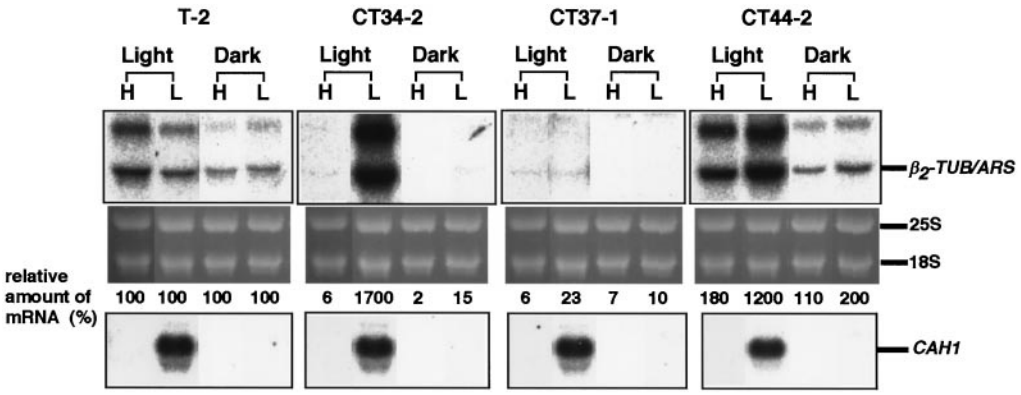

Figure 7. Light-responsive expression of the *ARS* reporter gene in transgenic *C. reinhardtii* cells. Cells were incubated under high- (H) or low-CO_2 conditions (L) either in the light or in the dark for 4 h. Ten micrograms of RNA from these cells was electrophoresed and blotted onto a membrane, and then hybridized with *ARS* cDNA (upper autoradiographs) or p-CA1-5′ (lower autoradiographs). Values of the relative amount of mRNA represent the percentage of the amount of the chimeric transcripts in CT34-2, CT37-1 and CT44-2 over the amount in the transformant T-2.

tured in the dark, the amount of β_2-*TUB/ARS* mRNA that accumulated under either high- or low-CO_2 conditions was less than one-tenth of that observed in T-2 cells. This result suggests that the silencer region represses transcription even under low-CO_2 conditions in the absence of light.

DISCUSSION

In this study, we fused a series of 5′-nested deletions of the region upstream of *CAH1* to an *ARS* reporter gene and identified regions essential for CO_2-responsive gene regulation (Fig. 1). As shown in Figure 2, quantification of arylsulfatase activity in transgenic *C. reinhardtii* revealed that the 692-bp region between −651 and +41 relative to the transcription start site is sufficient for full induction of the arylsulfatase activity under low-CO_2 conditions. Deletion of a 358-bp region (−651 to −294) resulted in a great reduction of the arylsulfatase activity but did not completely abolish CO_2-responsiveness (Fig. 2). Therefore, we expected that the 358-bp region might contain an enhancer element functioning under low-CO_2 conditions. However, this region could not enhance transcription of the β_2-tubulin minimal promoter, and instead was found to repress it (Fig. 6; CT37-1). One possible explanation for this result is that a silencer element located in this region works more dominantly than the enhancer element. No transformants expressing arylsulfatase activity were obtained when cells were transformed with pCAO5 or pCAO6 (Fig. 1). Although there is no evidence that these chimeric constructs were successfully incorporated into the *C. reinhardtii* genome, it is possible that they lack sequence elements essential to the arylsulfatase expression under low-CO_2 conditions, assuming that transformation frequency does not vary among different chimeric constructs.

The 543-bp upstream region between −651 to −109 without any promoter elements, CAAT-box, or TATA-box was sufficient to confer CO_2 and light responsiveness on the β_2-tubulin minimal promoter (Figs. 5 and 7). This 543-bp region was divided into two parts, a 358-bp silencer region from −651 to −294 and a 185-bp enhancer region from −293 to −109 (Fig. 6). The enhancer region activated the β_2-tubulin minimal promoter-driven *ARS* transcription in a light-dependent manner under low-CO_2 conditions (Fig. 7; CT44-2), while the silencer region repressed transcription in a manner independent of light illumination under high-CO_2 conditions and independent of the CO_2 level in the dark (Fig. 7; CT37-1). It is supposed that, under low-CO_2 conditions in the light, only the enhancer is able to achieve a sufficiently high level of gene activation to overcome the silencer effect and *CAH1* mRNA accumulates.

Previously, photosynthetic red light has been shown to be essential to activate the *CAH1* expression at the mRNA level (Dionisio-Sese et al., 1990). In addition to the photosynthesis-dependent processes, a blue-light-stimulated mechanism is thought to be involved in *CAH1* transcript regulation. Also, the *CAH1* expression is shown to depend on the phase of the cell cycle (Marcus et al., 1986) and circadian rhythm (Rawat and Moroney, 1995; Fujiwara et al., 1996). It is possible that these regulations might be mediated by the enhancer and silencer regions.

One question arises from comparison of the arylsulfatase expression patterns in cells harboring pCAO4 or pCT44. Why does deletion of the 358-bp region between −651 and −294 from pCT34 result in high-level arylsulfatase expression, as seen in cells harboring pCT44 under high-CO_2 conditions, whereas deletion of the same region from pCAO3 results in low-level expression, as seen in cells harboring pCAO4 under low-CO_2 conditions? A simple explanation is that region −109 to +41, which is present in pCAO4 but absent from pCT44, functions as a silencer under high-CO_2 conditions (CAO4 in Fig. 2). Consistent with this hypothesis, cells harboring pCT44, which contains neither the silencer region from −651 to −294 nor the other possible silencer region from −109 to +41, show no repression of arylsulfatase expression under high-CO_2 conditions, whereas cells harboring pCAO4, which contains the silencer region from −109 to +41, show normal repression of arylsulfatase expression.

The 543-bp region that is sufficient for CO_2-responsive transcriptional regulation was compared with upstream

regions of other low-CO_2-inducible genes, such as those that encode mitochondrial *β-CA* isozymes (Villand et al., 1997) and chloroplast envelope protein LIP-36 (Chen et al., 1997). A conserved CGCGCC sequence, which extends from −319 to −313 in the region upstream of *CAH*, was found in all of the genes. Additionally, two 12-mer sequences, GGGTTGAANTCCC (−553 to −541 in *CAH1*) and AACCCCNGNTGCA (−157 to −145), were also found in the upstream regions of *β-CA* genes. It has been reported that the expression of *β-CA1* and *β-CA2* is regulated in a manner similar to that of *CAH1*, not only being responsive to the external CO_2 concentration, but also light, acetate, and circadian rhythms (Eriksson et al., 1998). Interestingly, another conserved sequence, AGCGGCTCGC (−168 to −159 in *CAH1*), was found in the region upstream of *CAH2*, which is regulated by CO_2 and light in a manner opposite to that of *CAH1* (Fujiwara et al., 1990). Perhaps these sequence motifs function as CO_2- and/or light-responsive regulatory elements in *C. reinhardtii*.

Two sequence motifs that function in the promoters of higher plants were also detected. The first is a G-box-like sequence motif, CACGTTG, found at −310 to −304. The G-box is a ubiquitous, cis-acting element of plant genes to which bZIP proteins called G-box factors bind (Menkens et al., 1995). It is known that the G-box plays a role in the response of diverse promoters to factors such as light, anaerobiosis, and hormones including abscisic acid, ethylene, and auxin. The second is the sequence ATTTTCAC that is identical to a part of the ATCATTTTCACT light-responsive cis-element box III (Green et al., 1987). This motif lies within the enhancer region (−290 to −283). It is possible that this sequence is involved in the light-dependent transcriptional activation of *CAH1*.

Our results demonstrate that *CAH1* is regulated by enhancer and silencer elements in response to the external CO_2 level and light (Figs. 6 and 7). The presence of the enhancer element in the region upstream of *CAH1* and the fact that a mutant that does not induce *CAH1* under low-CO_2 conditions has been isolated (Fukuzawa et al., 1998) strongly suggest that regulatory mechanisms for transcriptional activation are functioning in *C. reinhardtii*. Considering the existence of *Chlorella ellipsoidea* mutants in which the CCM is not repressed under high-CO_2 conditions (Matsuda and Colman, 1996), negative regulatory mechanisms that repress CCM under CO_2-abundant conditions are operating in photosynthetic eukaryotes. In a cyanobacterium, *Synechococcus* sp. PCC 7942, positive and negative regulatory elements have also been shown in the promoter region of *cmpA*, which encodes a 42-kD low-CO_2-inducible protein (Ronen-Tarazi et al., 1995). These findings strongly suggest that CO_2 sensing and signaling mechanisms that control photosynthetic properties are commonly functioning in aquatic photosynthetic organisms.

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