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Structure and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii*

(photosynthesis/light requirement/CO_2-concentrating mechanism/zinc enzyme/glycoprotein)

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ABSTRACT **Two copies of structurally related genes (*CAH1* and *CAH2*) for carbonic anhydrase (EC 4.2.1.1) were found to be tandemly clustered on the *Chlamydomonas reinhardtii* genome. The previously isolated cDNA clones for carbonic anhydrase polypeptides were derived from the upstream gene, *CAH1*, which has 10 introns in its coding region. The downstream gene, *CAH2*, also has 10 introns, at positions identical to those of *CAH1*. Although amino acid sequences deduced from the two genes showed 91.8% identity, partial sequences of the authentic enzyme isolated from air-induced cells were identical only to those of the *CAH1* product. Northern hybridization using gene-specific probes showed that the level of 2.0-kilobase *CAH1* mRNA increased in response to a decrease in CO_2 concentration in the presence of light. The *CAH1* mRNA did not accumulate when CO_2 was lowered in the dark. In contrast, the level of 2.0-kilobase *CAH2* mRNA decreased in response to lowering of CO_2 and increased upon transfer to the high-CO_2 condition in light. The decrease of *CAH2* mRNA under the low-CO_2 condition was not observed in the dark. The fully induced mRNA level was much higher for *CAH1* than for *CAH2*. These results indicate that *CAH1* is a gene coding for the major periplasmic carbonic anhydrase whose level of transcript is rapidly induced under the low-CO_2 condition in the presence of light, and that *CAH2* may encode another periplasmic isozyme, which is made under the high-CO_2 condition.**

Carbonic anhydrase (CA; carbonate hydro-lyase, EC 4.2.1.1) catalyzes the reversible hydration of CO_2. In many microalgae, the activity of CA increases when high-CO_2 (4–5% CO_2) cells are transferred to low CO_2 (air level, 0.04% CO_2) (1). It has been suggested that CA, as well as a putative bicarbonate pump (1, 2), plays a role in increasing the affinity for inorganic carbon in photosynthesis of low-CO_2 cells. Although photosynthesis was not required for the induction of CA activity in *Chlorella vulgaris* 11h (3), both photosynthetic and nonphotosynthetic light reactions were required for the induction of CA activity in *Chlamydomonas reinhardtii* (4, 5). In the nonphotosynthetic light, blue light was effective for the induction (6). The increase of the enzyme activity, which was found mainly outside the plasma membrane (7), paralleled an increase of the CA polypeptide, a 35- to 37-kDa glycoprotein (8, 9).

A 4-kDa polypeptide is present in addition to the 35- to 37-kDa polypeptide (10). The subunit constitution of the holoenzyme is postulated to be a heterotetramer consisting of two large and two small subunits linked by disulfide bonds. Moreover, cDNA clones encoding CA polypeptides were isolated by using oligonucleotide probes based on amino acid sequences of the two kinds of subunits (11). Nucleotide sequence analysis revealed that the large and the small subunits are cotranslated as a 377-amino acid precursor polypeptide (41,626 Da) with a signal peptide of 20 amino acids from a 2.0-kilobase (kb) mRNA transcript. The level of the 2.0-kb CA mRNA was induced by lowering the environmental CO_2 concentration in light.

Mammalian CA comprises seven types of isozymes (I–VII) (12). By comparing the three-dimensional structures of human CAI and CAII, as determined by x-ray crystallography, the amino acid residues essential for the enzyme activity have been deduced (12, 13). The human genes for CAI, CAII, and CAIII (14–16) are located on chromosome 8 and are closely linked (17). The genes for CAII of human, mouse, and chicken are interrupted by six introns at the same positions.

In this paper, we report the nucleotide sequences of two CA genes in *C. reinhardtii*.‡ Differential expression of the two CA genes was examined at the mRNA level with gene-specific probes.

MATERIALS AND METHODS

Cells and Culture Conditions. *C. reinhardtii* cells (Dangeard C-9 mt$^-$) were cultured at 30°C in 0.3× HSM medium (34) by aeration with air containing 5% CO_2, to obtain high-CO_2 cells, or with ordinary air (0.04% CO_2), to obtain low-CO_2 cells, both under continuous illumination at 18 W/m^2 (5).

Construction and Screening of Genomic DNA Library. Total DNA was isolated from *Chlamydomonas* cells by CsCl ultracentrifugation (18). The genomic DNA library was constructed in λ phage EMBL3 by using *Chlamydomonas* DNA partially digested with *Sau*3A1 (19). Based on partial amino acid sequences of CA subunits and the most probable codon usage in *Chlamydomonas* genes, two oligonucleotide probes, Pr68 and Pr56, were synthesized (11). The sequence of Pr68 was complementary to a putative mRNA sequence encoding part of a CNBr-cleaved peptide fragment derived from the large subunit. The sequence of Pr56 was complementary to a putative mRNA sequence encoding a part of the small subunit. Plaque hybridization and washing were carried out as described (11).

DNA Sequence Analysis. Isolated genomic DNA fragments in EMBL3 were subcloned into plasmid pUC19 and phages M13mp18 or M13mp19. Stepwise deletion clones were obtained by exonuclease III digestion (20) and used in sequencing (21).

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Abbreviations: CA, carbonic anhydrase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. D90206 (*CAH1*) and D90207 (*CAH2*)].

RNA Blot Hybridization. Equal amounts of total RNA (10 μg) were denatured and electrophoresed in formaldehyde-containing 1% agarose gel (18), blotted to nylon membranes (Zeta-Probe; Bio-Rad), and probed by hybridization with terminally ^{32}P-labeled 40-meric oligodeoxynucleotides PrCAH1 and PrCAH2, which are specific to *CAH1* and *CAH2* genes, respectively. The sequences of PrCAH1 and PrCAH2 were 5′-GCCGTGCCGACGGTGGTAGCGTGAC-TAACTACTGGGAAGT-3′ and 5′-CAGTGCTCACATAG-TAGTTTCGAATTCTGCCAATCCTGTC-3′, respectively. An RNA ladder (BRL) was used as size markers.

RESULTS

Isolation of Genomic Clones Encoding CA Genes. To determine the copy number of CA genes, genomic Southern hybridizations were carried out using oligonucleotide probes Pr68, specific for the large subunit, and Pr56, specific for the small subunit. Two positively hybridized bands were detected when the *Chlamydomonas* genomic DNA was digested with *Hin*cII or *Pst* I. The Pr68 probe hybridized with 4.0-kb and 0.8-kb *Hin*cII fragments and 1.2-kb and 0.9-kb *Pst* I fragments (Fig. 1*A*). The Pr56 probe hybridized with 4.0-kb and 2.0-kb *Hin*cII fragments and 3.5-kb and 2.3-kb *Pst* I fragments (Fig. 1*B*). It was therefore predicted that two CA genes existed in the *Chlamydomonas* genome.

Genomic clones were isolated by screening the EMBL3 genomic DNA library with ^{32}P-labeled Pr68 probe. DNA samples purified from these clones were digested with *Hin*cII or *Pst* I and hybridized with ^{32}P-labeled Pr68. From the isolated recombinant phages F1 and F9, two hybridized bands of 4.0 kb and 0.8 kb were produced by *Hin*cII digestion and two bands of 1.2 kb and 0.9 kb by *Pst* I digestion, as in the case of genomic Southern hybridization (data not shown). Therefore, the two CA genes appeared to be close to each other in the *Chlamydomonas* genome, since both genes were cloned in the recombinant phages F1 and F9. Restriction mapping and partial sequence analysis of insert DNA fragments in the two phages revealed that the inserts in the two phages overlapped each other and that two CA genes were clustered tandemly in the *Chlamydomonas* genome (Fig. 2*A*). The 5′ upstream gene for CA was designated *CAH1*, and the 3′ downstream gene *CAH2*, respectively. Positions that corresponded to oligonucleotide probes Pr68 and Pr56 were located in the coding regions of the two genes.

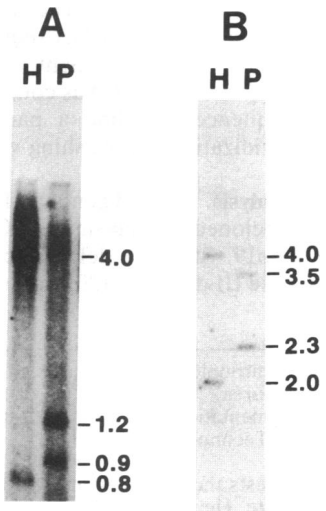

FIG. 1. Southern blot analysis of *Chlamydomonas* DNA digested with *Hin*cII (lanes H) or *Pst* I (lanes P) and probed with ^{32}P-labeled Pr68 (*A*) and Pr56 (*B*). Numbers indicate molecular sizes in kilobases.

Nucleotide Sequence Analysis of the Upstream Gene, *CAH1*. To determine the structure of the *CAH1* gene, the nucleotide sequence of the 5009-bp region from the *Eco*RI site to the *Ava* I site in the phage F1 was determined on both strands by using overlapping subclones. Since the sequences of parts of *CAH1* were identical with those of corresponding parts of cDNA gtCA3 encoding a 377-amino acid CA precursor polypeptide (11), it was concluded that the cDNA was derived from the 5′ upstream gene *CAH1*. The first adenine of the translation initiation codon (ATG) was numbered as +1 in *CAH1*. The translation stop codon (TAA) was found at positions 3433–3435. *CAH1* consisted of 11 exons and 10 introns, and the length of the coding region, including introns, was 3432 bp (Fig. 2*B*). The coding region for the large subunit was interrupted by 9 introns, and that for the small subunit by the 10th intron. In the first exon, a hydrophobic signal peptide of 20 amino acid residues and the NH_2-terminal 12-amino acid residues of the large subunit were encoded. Two of the three histidine residues (His-163 and His-165) that are predicted to coordinate to a zinc ion (11) were encoded in exon 7, while the third (His-182) was encoded in exon 8. Exon 10 encoded the putative COOH-terminal region of the large subunit and the NH_2-terminal region of the small subunit. The 5′- and 3′-terminal sequences of the 10 introns in *CAH1* had the conserved sequences GT(G or A)(A or C)G at the 5′ donor site and CAG at the 3′ acceptor site, which were similar to the eukaryotic consensus GT/AG rule (22).

The 5′ terminus of the cDNA fragment was localized at position −30. Putative promoter elements—a Hogness–Goldberg TATA box and a CAAT box (22)—were found at positions −71 to −67 and −150 to −147, respectively. The poly(A) addition site was at positions 4223–4228, located 788 bp downstream from the stop codon. The sequence TGTAA, which is thought to be a polyadenylylation signal conserved in *Chlamydomonas* genes (23–26), was found 18 bp upstream from the poly(A) site. At positions 4341–4390, alternating A and C residues were found 25 times.

Nucleotide Sequence Analysis of the Downstream Gene, *CAH2*, and Peptide Sequence Comparison of the Two Gene Products. Since the insert DNA of phage F1 did not contain the complete region of the *CAH2* gene, the overlapping phage F9 was also used for sequence determination. The 3106-bp region from a *Pst* I site to another *Pst* I site in the F1 phage DNA and the 1931-bp region from the *Hin*cII site to the *Hae* III site in the F9 phage DNA were sequenced as indicated in Fig. 2*A*. As the sequences between the *Hin*cII site and the *Pst* I site in the two phages were identical, the two sequence files were connected into a single sequence file of the 4858-bp *Pst* I–*Hae* III region. By comparing the sequence of the *Pst* I–*Hae* III region with that of *CAH1* and considering conserved sequence of the splice junction described above, exons and introns of *CAH2* were predicted (Fig. 2*C*). The first adenine of the ATG codon corresponding to the translation initiation codon of the *CAH1* gene was numbered as +1. The predicted length of the *CAH2* coding region, including introns, was 3309 bp. Nucleotide sequence identity of the coding region of *CAH2* with that of *CAH1* was 93.6%, and the codon usage of *CAH2* was highly biased as in the case of *CAH1*. The *CAH2* gene was interrupted by 10 introns in the coding sequence at the same positions as in the case of *CAH1* (Fig. 3). When predicted intervening sequences of *CAH2* were compared with those of *CAH1*, introns 1, 8, and 10 showed no significant sequence similarity except for those 5′- and 3′-terminal regions. In contrast, other introns of *CAH2* shared sequence similarities with corresponding *CAH1* introns.

Two pairs of eukaryotic consensus sequences for transcription initiation were found upstream from the translation initiation site: possible Hogness–Goldberg TATA boxes, TAAAA and TATATA, were localized at positions −63 and

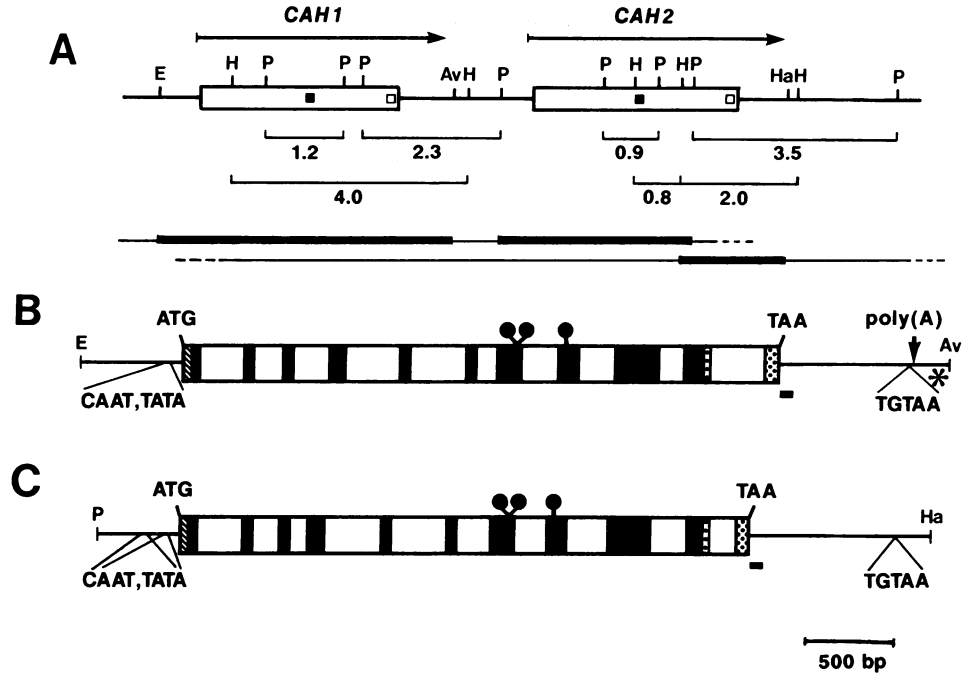

FIG. 2. Organization of *Chlamydomonas* CA genes. (*A*) Coding regions, including introns, are shown by open boxes. Filled and open squares indicate the positions to which Pr68 and Pr56 hybridize, respectively. Positions of restriction sites are indicated (Av, *Ava* I; E, *Eco*RI; H, *Hin*cII; Ha, *Hae* III; P, *Pst* I), and lengths (kb) of selected fragments are shown. Thick parts of the lower two lines indicate the regions sequenced using F1 (upper of the two) and F9 (lower) phage inserts. (*B* and *C*) Structure of *CAH1* and *CAH2*, respectively. Protein-coding regions for signal peptides, large subunit, and small subunit are indicated by hatched, filled, and stippled boxes, respectively. Filled circles indicate the positions of zinc-liganded histidine residues. Positions of gene-specific probes are shown by bars just downstream from the TAA termination codons. An asterisk indicates alternating A and C residues repeated 25 times. bp, Base pairs.

−151, respectively. Two CAAT boxes were located beginning 119 and 246 bp upstream from the initiation codon. A TGTAA sequence, which is predicted to be a polyadenylylation signal in *Chlamydomonas* genes (23–26), was also found, 861 bp downstream from the stop codon in *CAH2*.

Sequence analysis of the 3′ (downstream) gene, *CAH2*, revealed that it may encode a 380-amino acid precursor polypeptide that shows 91.8% amino acid sequence identity with the *CAH1* gene product (Fig. 3). Partial amino acid sequences corresponding to probes Pr68 and Pr56 were found at positions 147–169 and 355–373 (based on deduced amino acid sequence of *CAH2*), respectively. The deduced amino acid residues Ser-152, Thr-355, Thr-356, and Ala-366 in the *CAH2* gene product differed from the corresponding residues of the authentic CA polypeptide isolated from air-induced cells. However, three zinc-liganded histidine residues (His-163, His-165, and His-182; based on *CAH2* numbering) and those predicted to form the hydrogen-bond network to zinc-bound solvent molecules (Ser-68, His-112, Gln-115, Gln-161, Glu-169, His-170, Glu-180, Tyr-255, Thr-260, Thr-261, Trp-270, Asn-362, and Arg-364) in mammalian CA isozymes (12, 13) were conserved in the *CAH2* gene product. Three putative asparagine-linked glycosylation sites (33) were localized at Asn-101, Asn-135, and Asn-297 as in the case of the *CAH1* gene.

Northern Blot Analysis of Transcripts from *CAH1* and *CAH2*. To clarify the expression pattern of the two genes, levels of transcripts in response to changes of CO_2 concentration and light were determined by Northern hybridization using the gene-specific probes PrCAH1 and PrCAH2. The nucleotide sequences of the two probes were complementary to those of parts of mRNA transcripts from the two genes

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CAH1  MARTGALLLVALALAGCAQACIYKFGTSPDSKATVSGDHWDHGLNGENWEGKDGAGNAWV  60
CAH2  MARTGALLLAALALAGCAQACIYKFGTSPDSKATHTGDHWDHSLNGENWEGKDGAGNPWV  60

      CKTGRKQSPINVPQYQVLDGKGSKIANGLQTQWSYPDLMSNGTSVQVINNGHTIQVQWTY  120
      CKTGRKQSPINVPQYHVLDGKGSKIATGLQTQWSYPDLMSNGSSVQVINNGHTIQVQWTY  120

      NYAGHATIAIPAMHNQTNRIVDVLEMRPNDAADRVTAVPTQFHFHSTSEHLLAGKIYPLE  180
      DYAGHATIAIPAMRNQSNRIVDVLEMRPNDASDRVTAVPTQFHFHSTSEHLLAGKIFPLE  180

      LHIVHQVTEKLEACKGGCFSVTGILFQLDNGPDNELLEPIFANMPSREGTFSNLPAGTTI  240
      LHIVHKVTDKLEACKGGCFSVTGILFQLDNGPDNELLEPIFANMPTREGTFTNLPAGTTI  240

      KLGELLPSDRDYVTYEGSLTTPPCSEGLLWHVMTQPQRISFGQWNRYRLAVGLKECNSTE  300
      KLGELLPSDRDYVTYEGSLTTPPCSEGLLWHVMTQPQRISFGQWNRYRLAVGEKECNSTE  300

      T--A-ADAG-HHHHHRRLLHNHAHLEEVPAATSEPKHYFRRVMLAESANPDAYTCKAVAF  356
      TDAAHADAGHHHHHHRRLLHNHAHLEEVPAATSEPKHYFRRVM-EETENPDAYTCTTVAF  359

      GQNFRNPQYANGRTIKLARYH  377
      GQNFRNAQYANGRTIKLARYE  380
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FIG. 3. Comparison of amino acid sequences deduced from CA genes *CAH1* and *CAH2*. Asterisks indicate identical amino acid residues, and filled squares show possible asparagine-linked glycosylation sites. Putative zinc-liganded histidines and residues forming hydrogen-bond network to zinc-bound solvent molecules are depicted by filled and open circles, respectively. Arrows indicate the junction site between the signal peptide and the large subunit and between the large and the small subunit. Insertion sites of introns are indicated by open triangles.

corresponding to positions just after translation stop codons and had no significant homology to each other. *CAH1* and *CAH2* mRNA levels were assayed during the period when CA activity and protein accumulation increased (9)—that is, after the culture aeration was changed from CO_2-enriched air (5%) to air (0.04%), under continuous illumination (Fig. 4*A*). The PrCAH1 probe specifically hybridized with mRNA of the same length, 2.0 kb, as was found with the ^{32}P-labeled cDNA probe (11). No accumulation of 2.0-kb transcript hybridizing with the PrCAH1 was observed under the high-CO_2 condition (lane 1), even when the autoradiography was prolonged. The level of *CAH1* mRNA increased within 1 hr and attained the maximum 2 hr after the CO_2 concentration was lowered (lanes 1–5). In contrast, accumulation of the *CAH2* mRNA, which was detected as a 2.0-kb RNA at a much lower level than that of the *CAH1* transcript in the fully induced conditions, was repressed within 1 hr after CO_2 was lowered (lanes 6–10). However, a small accumulation of the 2.0-kb mRNA was still observed 6 hr after air induction.

When low-CO_2 cells were aerated by CO_2-enriched air (5%) in light, the level of *CAH1* mRNA decreased to an undetectable level within 1 hr (Fig. 4*B*, lanes 1–5), as shown with the cDNA probe. On the other hand, the level of *CAH2* mRNA increased within 1 hr and reached a maximum 2 hr after the CO_2 level was increased (lanes 6–10). These results indicate that the expression patterns of *CAH1* are essentially the same as those observed using the cDNA probe, and that levels of the *CAH2* transcript are regulated in the reverse manner as compared with *CAH1*.

The *CAH1* mRNA did not accumulate when high-CO_2 cells were transferred to the low-CO_2 condition in the dark (Fig. 4*C*, lane 2). Similarly, addition of 10 μM DCMU to air-adapting cells inhibited the accumulation of the *CAH1* mRNA (lane 3). These results indicate that light, low CO_2, and photosynthesis are essential for the accumulation of the *CAH1* transcript. On the other hand, the level of the *CAH2* mRNA was higher when CO_2 was lowered in the dark (lane 7) than when it was lowered in light (lane 6). The level of the *CAH2* mRNA was not affected by the addition of 10 μM DCMU (lane 8). Addition of actinomycin D (20 μg/ml), which preferably inhibits nuclear transcription, completely stopped the accumulation of both *CAH1* and *CAH2* mRNA transcripts (lanes 4 and 9). In contrast, addition of rifampicin (80 μg/ml), a transcriptional inhibitor specific for prokaryotic RNA polymerases, did not have any effect on the accumulation of the two CA mRNA species (lanes 5 and 10). These results confirm that the two CA genes are encoded by the nuclear genome. In lanes 1 and 5 in Fig. 4*C*, additional bands of 4.3 kb were observed, since the contact period of autoradiography was longer than in Fig. 4 *A* and *B*. The 4.3-kb band corresponded in size to the precursor mRNA including introns predicted from the structure of the upstream gene, *CAH1*.

DISCUSSION

That nucleotide sequence identity of the coding regions between *CAH1* and *CAH2* was 93.6% and that insertion sites of introns in the coding regions of *CAH2* were identical with those of *CAH1* suggest that the two CA genes encoded by the *Chlamydomonas* genome have evolved from an ancestral CA gene containing 10 introns. In animals, CA genes appear to have 6 introns at almost the same positions (27, 28) except for the human CAI gene (14). In contrast, both CA genes of *Chlamydomonas* have 10 introns at the positions different from those of animals. Moreover, the *Chlamydomonas* CA genes (≈4 kb) are smaller than those of animal CAII (≈17 kb; refs. 27 and 28) and human CAIII (≈10 kb; ref. 16).

Although the sequences upstream from the two CA genes diverged extensively except for a 12-bp 5′ untranslated region just before the initiation codon ATG, a 10-bp conserved sequence motif (AGCGGCTCGC) occurred 210 bp and 188 bp upstream from the initiation codons of *CAH1* and *CAH2*, respectively. An almost identical sequence motif (AGCGGCTCTC) was found 880 bp upstream from the transcription initiation site in *Chlamydomonas rbcS2*, which is the upstream gene of the two genes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (26). This sequence block may function as a cis-acting element in the regulation of expression of CA genes and *rbcS2*, but no functional evidence for this hypothesis has been obtained. Beginning 118 bp downstream from the poly(A) site of *CAH1*, alternating A and C residues occurred 25 times, which may have the potential to assume a Z-DNA conformation (29). In the case of *Chlamydomonas rbcS1*, G and T residues, complementary to A and C, are repeated eight times in the 3′ untranslated region (26). These repeated sequences may function as a regulatory element by forming a Z-DNA conformation.

Although partial sequences of the authentic enzyme from low-CO_2 cells were identical only to those of the *CAH1* gene product, the amino acid sequence deduced from *CAH2* had the following common characteristics with that from *CAH1* (Fig. 3). (*i*) Each sequenced region encoded a polypeptide consist-

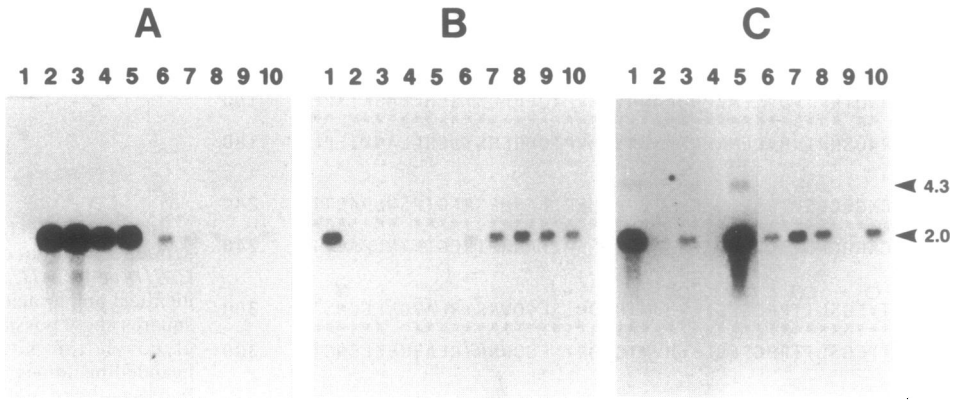

FIG. 4. Northern blot analysis of *Chlamydomonas* RNA with gene-specific probes PrCAH1 (lanes 1–5) and PrCAH2 (lanes 6–10). (*A*) High-CO_2 cells (lanes 1 and 6) were transferred to low CO_2 in light for 1 hr (lanes 2 and 7), 2 hr (lanes 3 and 8), 4 hr (lanes 4 and 9), or 6 hr (lanes 5 and 10). (*B*) Low-CO_2 cells (lanes 1 and 6) were kept in high CO_2 for 1 hr (lanes 2 and 7), 2 hr (lanes 3 and 8), 4 hr (lanes 4 and 9), or 6 hr (lanes 5 and 10) in light. (*C*) To show effects of light and inhibitors on CA transcripts, high-CO_2 cells were kept in low CO_2 for 4 hr in light (lanes 1 and 6) or in the dark (lanes 2 and 7) or in the presence of light and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (lanes 3 and 8), 20 μg of actinomycin D per ml (lanes 4 and 9), or 80 μg of rifampicin per ml (lanes 5 and 10). Transcript lengths are indicated in kilobases.

ing of a hydrophobic signal peptide, a large subunit, and a small subunit. (*ii*) Amino acid residues that are necessary for zinc folding were conserved except for 3 or 2 amino acid residues found in human isozymes (Tyr-7, His-67, and His-200 in CAI; Tyr-6 and Asn-66 in CAII; ref. 11). (*iii*) Three putative asparagine-linked glycosylation sites were located at Asn-101, Asn-135, and Asn-297. These similarities suggest that the *CAH2* product has CA activity and that it may be localized in the periplasmic space of *Chlamydomonas* cells, as in the case of the major CA polypeptide, which is the *CAH1* product. Since the existence of internal CA polypeptides has been reported in addition to the external CA (30), the possibility that the *CAH2* product may be an internal CA polypeptide cannot be excluded. This possibility is unlikely, however, because of similarities of the *CAH2* and *CAH1* products.

Major structural differences between the two gene products are as follows. (*i*) Additional amino acid residues, Asp-302, Ala-303, His-305, and His-310, were observed in the *CAH2* gene product. (*ii*) In the *CAH2* product, Leu-340, which is located just before the NH_2 terminus of the small subunit in the *CAH1* precursor polypeptide, was missing. Thus the processing site between the large and small subunits in the *CAH2* product may differ from that in the *CAH1*.

The two genes were differentially regulated at the level of transcript according to changes of the CO_2 concentration and light. The expression of the upstream gene, *CAH1*, was repressed in high CO_2 (5%) and activated by lowering the CO_2 to 0.04% (air level) in light. In contrast, the expression of the downstream gene, *CAH2*, was activated by high CO_2 and repressed by low CO_2 in light. However, low-CO_2-induced repression of *CAH2* was not observed in the dark. Therefore, we conclude that light is essential for *CAH1* expression, whereas light has an inhibitory effect on *CAH2* expression. Differentially regulated expression has also been reported for other genes of *Chlamydomonas* cells. Two *rbcS* genes are tandemly clustered at a single locus and are differentially regulated by light and/or carbon source (26).

In our previous paper on cDNA cloning (11), no accumulation of CA transcript, including *CAH2* transcript, was observed in the dark by Northern blot analysis using the cDNA probe. This can be explained by considering that the cDNA probe has a long 3′ noncoding region that has no sequence similarity with the putative *CAH2* transcript. When oligonucleotide probe Pr68, which hybridizes with *CAH1* mRNA as well as *CAH2* mRNA, was used in Northern hybridization, a positively hybridized band was observed in the dark, but the level of CA transcript was apparently much lower than in low-CO_2-induced cells in light (31). Bailly and Coleman (32) reported that the CA gene existed as a single copy on the *Chlamydomonas* genome and showed the physical map of a genomic clone that was different from that shown in Fig. 2*A*. These differences may have been caused by the use of different *Chlamydomonas* strains, C-9 and 2137. They also reported that a significant level of CA transcript was detected in the dark. It is possible that the transcript of the strain 2137 in the dark is the *CAH2* transcript, since the *CAH2* mRNA of the strain C-9 was expressed under the low-CO_2 condition in the dark (Fig. 4*C*, lane 7).

Although the activity and polypeptide accumulation of CA were low under the high-CO_2 condition, these could still be detected (31). In this condition, no *CAH1* mRNA was detected in the cells and only the *CAH2* mRNA was actually observed. These facts can be explained by assuming that the *CAH2* mRNA is translated into enzymatically active CA polypeptides in the high-CO_2 condition.

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