CO_2 Sensing at Ocean Surface Mediated by cAMP in a Marine Diatom[1]

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Marine diatoms are known to be responsible for about a quarter of global primary production and their photosynthesis is sustained by inorganic carbon-concentrating mechanisms and/or C_4 metabolism. Activities of the inorganic carbon-concentrating mechanism are attenuated under enriched [CO_2]; however, impacts of this factor on primary productivity and the molecular mechanisms of CO_2 responses in marine diatoms are unknown. In this study, transgenic cells were generated of the marine diatom *Phaeodactylum tricornutum* by the introduction of a β-glucuronidase reporter gene under the control of an intrinsic CO_2-responsive promoter, which is the sequence between -80 to $+61$ relative to the transcription start site of a chloroplastic-carbonic anhydrase gene, *ptca1*, obtained from *P. tricornutum*. The activity of the *ptca1* promoter was effectively repressed in air-level CO_2 by treating cells with a 1.0 mM cAMP analog, dibutyryl cAMP, or a cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. Deletion of the intrinsic cAMP-response element from the *ptca1* promoter caused a lack of repression of the reporter gene *uidA*, even under elevated [CO_2] and a null phenotype to the strong repressive effects of dibutyryl cAMP and 3-isobutyl-1-methylxanthine on the *ptca1* promoter. Deletion of the cAMP-response element was also shown to cause derepression of the *uidA* reporter gene in the dark. These results indicate that the cytosolic cAMP level increases under elevated [CO_2] and represses the *ptca1* promoter. This strongly suggests the participation of cAMP metabolism, presumably at the cytosolic level, in controlling CO_2-acquisition systems under elevated [CO_2] at the ocean surface in a marine diatom.

Marine diatoms are responsible for one-half of primary productivity in the ocean and hence play a key role in global cycles of carbon and other inorganic nutrients (Tréguer et al., 1995; Falkowski et al., 2000). [CO_2] dissolved in seawater is limited under the present atmospheric pCO_2 (below 15 μM at 20°C) that is much lower than the K_m[CO_2] of Rubisco in diatom species (Badger et al., 1998). This implies that marine diatoms need active uptake and accumulation systems for dissolved inorganic carbon (DIC) to support their photosynthesis. There is a substantial body of evidence that the operation of the inorganic carbon-concentrating mechanism (CCM) confers on marine diatom cells high-affinity photosynthesis for DIC (Colman and Rotatore, 1995; Johnston and Raven, 1996; Matsuda et al., 2001), which is due to the operation of active uptake of both CO_2 and HCO_3^- (Colman and Rotatore, 1995; Johnston and Raven, 1996; Matsuda et al., 2001). The activity of the CCM is suppressed under CO_2-enriched conditions, whereas it is induced in CO_2-limiting conditions; this regulation is due to CO_2 sensing by algal cells and induction of the CCM facilitates an ample supply of CO_2 to Rubisco even under extreme [CO_2] limitation (Badger et al., 1980, 1998; Kaplan et al., 1980; Miller et al., 1990; Colman and Rotatore, 1995; Johnston and Raven, 1996; Tortell et al., 1997; Moroney and Somanchi, 1999; Matsuda et al., 2001).

A number of physiological evidences have shown major differences in the mode of regulation in CCM expression between cyanobacteria and eukaryotic algae. The differences are particularly evident with respect to O_2 dependency and the critical determinant for the extent of CCM expression. Decrease in [O_2] from 21% to 2.6% during acclimation of the cyanobacterium *Anabaena variabilis* to low [CO_2] caused a significant delay of CCM induction (Marcus et al., 1983). This is in complete agreement with the recent finding that transcription of most CCM components requires O_2 for full induction in *Synechococcus* PCC7942 under CO_2 limitation (Woodger et al., 2005). In contrast, it was clearly demonstrated in the green alga *Chlamydomonas reinhardtii* that inductions of CO_2-regulated genes and the CCM are completely independent of O_2 concentrations but that CO_2 alone is important for this process (Vance and Spalding, 2005). The absence of O_2 effect on CCM expression was also shown in the green alga *Chlorella ellipsoidea* (Matsuda et al., 1998). These investigations clearly revealed at least two

[1] This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT; Grant-in-Aid for Scientific Research B no. 18310014 to Y.M.); by the Showa-Shell Sekiyu Environmental Research Foundation (to Y.M.); by the Program for Research on Halophilic Organism of the Salt Science Research Foundation (grant no. 05B02 to Y.M.); and by the University-Industry Joint Research Project from MEXT (to Kwansei-Gakuin University, Nano-Biotechnology Research Centre).

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www.plantphysiol.org/cgi/doi/10.1104/pp.106.086561

distinct lines of the CO_2-response system in CCM regulation. Close relations of the bacterial-type CCM regulation with $[O_2]$ probably reflect the metabolic state in photosynthesis induced by changing the CO_2 to O_2 ratio (Kaplan and Reinhold, 1999), whereas the data obtained in green algae suggest the occurrence of the relatively direct perception of CO_2 signal in the eukaryotic CCM regulations. In cyanobacteria, it is assumed therefore that low CO_2 signal will disappear or be diminished after cells have developed an effective level of the CCM during acclimation to low CO_2. This is supported by the finding that transcription of CCM components occurs in response to a transient decrease in internal [DIC] at the initial stage of acclimation to low CO_2 but was suppressed according to the development of the internal DIC pool at the late stage of acclimation to low CO_2 in *Synechococcus* PCC7942 (Woodger et al., 2005). In sharp contrast, it was clearly demonstrated that $[CO_2]$ in the bulk medium is the critical determinant for the extent of CCM expression, but other DIC species and temporary decrease in internal DIC are not responsible for CCM regulation in the green alga *C. ellipsoidea* (Matsuda and Colman, 1995). The occurrence of $[CO_2]$ as a critical determinant for the CCM expression level has also been reported in the green algae *C. reinhardtii* (Bozzo and Colman, 2000; Vance and Spalding, 2005) and *Chlorella kessleri* (Bozzo et al., 2000), and the marine diatom *Phaeodactylum tricornutum* (Matsuda et al., 2001).

Light is a crucial but not absolute factor to develop a full expression of the CCMs in cyanobacteria and eukaryotic algae. Dependence on light seems to vary considerably among species, and in green algae it is often very weak (i.e. significant levels of CCM expression occurred in the dark in air; Matsuda and Colman, 1995; Bozzo and Colman, 2000). In *C. reinhardtii*, regulation of the CO_2-responsive gene *Cah1* was shown to be regulated by both silencer and enhancer in its promoter region in response to changing the ambient $[CO_2]$, and the absence of light constitutes a repressive signal to the *Cah1* promoter via the silencer region (Kucho et al., 1999). These observations strongly suggest the occurrence of cross talk between light and CO_2 signals in CCM regulation.

CO_2-sensing mechanisms, as described above, have been studied in a limited number of algae, primarily freshwater and soil species (Matsuda and Colman, 1995; Rawat and Moroney, 1995; Badger et al., 1998; Eriksson et al., 1998; Kaplan and Reinhold, 1999; Kucho et al., 1999; Bozzo and Colman., 2000; Miura et al., 2004; Vance and Spalding, 2005; Woodger et al., 2005), but little molecular information is available on marine eukaryotic algae. CO_2 is a redundant molecule that influences a variety of physiological processes in microbes, plants, and animals, and, as a precedent, the initial process of sensing $[CO_2]$ has been found in a mammalian tissue. In rat testis, the activity of soluble adenylyl cyclase (sAC) was shown to be stimulated directly by the addition of HCO_3^- and Ca^{2+} (Chen et al., 2000; Jaiswal and Conti, 2003), with which ejaculated spermatozoa could undergo a series of HCO_3^--induced initial activation processes (Chen et al., 2000). Since this gene for sAC was found to be evolutionally conserved from cyanobacteria to mammals (Chen et al., 2000), the CO_2-sensing system mediated by cytosolic cAMP levels was proposed to be a general mechanism that might operate in the regulation of the CCM in cyanobacteria (Chen et al., 2000). In fact, several cyanobacterial sACs, CyaC, CyaB1, and Cya1, are stimulated or inhibited by the addition of HCO_3^- (Chen et al., 2000; Masuda and Ono, 2005). However, DIC species, critical for the stimulation of sACs, was not clear in these studies. Recently, a sAC, Cya1 in *Synechocystis* PCC6803, was shown to be stimulated by CO_2 rather than HCO_3^- (Hammer et al., 2006). It is thus possible that CO_2-responsive events, including CCM expression, might be controlled not only by HCO_3^- but also directly by $[CO_2]$, in some cases via the second-messenger cAMP. At present, however, no relation among sACs, cAMP, and CCM regulation has been reported either in cyanobacteria or in eukaryotic algae. In marine diatoms, some putative sACs are found in the genome database of *Thalassiosira pseudonana*, but these genes are not related to CO_2/HCO_3^- responses.

A β-carbonic anhydrase in the marine diatom *P. tricornutum* (PtCA1), thought to be one of the crucial chloroplastic components of the CCM (Satoh et al., 2001; Tanaka et al., 2005), is known to be regulated strictly by the ambient $[CO_2]$ and light (Harada et al., 2005). A promoter region of the PtCA1 gene (*ptca1*) was isolated previously, and it was demonstrated that the critical CO_2-response sequence was located downstream -70 bp relative to the transcription start site (Harada et al., 2005). This relatively short core regulatory sequence comprises two putative cAMP-response elements, CRE1 at -70 to -63 and CRE2 at -21 to -14, and a putative p300-binding element at -52 to -46 relative to the transcription start site (Harada et al., 2005; Fig. 1). In this study, the activity of this core regulatory region was investigated by replacing the *ptca1* gene with the β-glucuronidase (GUS) reporter gene *uidA*.

RESULTS

Deletion and Substitution Assays of the *ptca1* Promoter with the GUS Reporter Gene *uidA*

The upstream sequence from -80 to $+61$ relative to the transcription start site of the *ptca1* gene (Fig. 1), which includes the CO_2-responsive core regulatory region of the *ptca1* promoter, P*ptca1* (Harada et al., 2005), was ligated to the GUS reporter gene *uidA*. Prior to the deletion experiment, GUS activities in two independent *P. tricornutum* clones, which were transformed with the *uidA* reporter gene under the control of P*ptca1*, were compared with *uidA* transcript levels (Fig. 2). GUS activities increased 4.0- to 5.3-fold in the

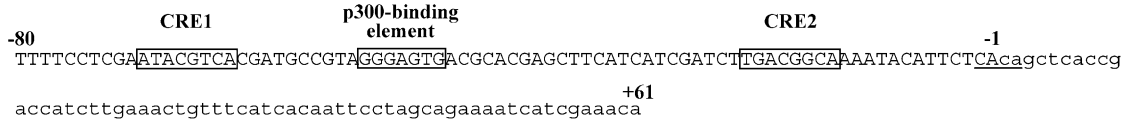

Figure 1. The core regulatory region of P*ptca1*, which is −80 to +61 bp relative to the transcription start site. Three putative cis-elements, CRE1, p300-binding element, and CRE2, are boxed. The putative initiator sequence is underlined. The upstream and downstream sequences of the transcription start site are indicated with uppercase and lowercase letters, respectively.

air-grown transformants as compared to those in high-CO_2-grown transformants (Fig. 2A). Transcript levels of *uidA* were detected at levels corresponding to GUS activities irrespective of growth conditions and clones (Fig. 2B), indicating that GUS activity can be a direct indicator for activities of P*ptca1*.

To identify the critical CO_2-responsive cis-elements, a series of 10-bp deletions or base substitutions were carried out to the core regulatory region of the *ptca1* promoter, P*ptca1* (Fig. 3, left). Manipulated promoter constructs were fused with the reporter gene *uidA* (Fig. 3, left) and were introduced into *P. tricornutum* cells. A single deletion of the region −71 to −62 or the region −52 to −42, which, respectively, includes CRE1 or the putative p300-binding region, resulted in the apparent derepression of GUS expression in high-CO_2-grown transformants (Fig. 3, B and D). GUS expression ratios (air/5% CO_2) were reduced significantly to less than 2.0 (Fig. 3, B and D), and, thus, these transformants became weakly responsive to CO_2 with nearly constitutive expressions of GUS. A similar regulatory profile of *uidA* was also seen in transformants by deleting both CRE1 and CRE2 (−21 to −13; Fig. 3H). Derepression of *uidA* was again the case when regions containing these elements were substituted with the respective antisense sequences (Fig. 3, I and K). A similar derepression of *uidA* under high CO_2 was also observed by deleting the p300-binding element (Fig. 3D). On the other hand, a single deletion or substitution of the CRE2 region did not cause a reduction in the CO_2 sensitivity of *uidA* expression (Fig. 3, G and J). Deletion of CRE2 appeared rather to enhance the expression

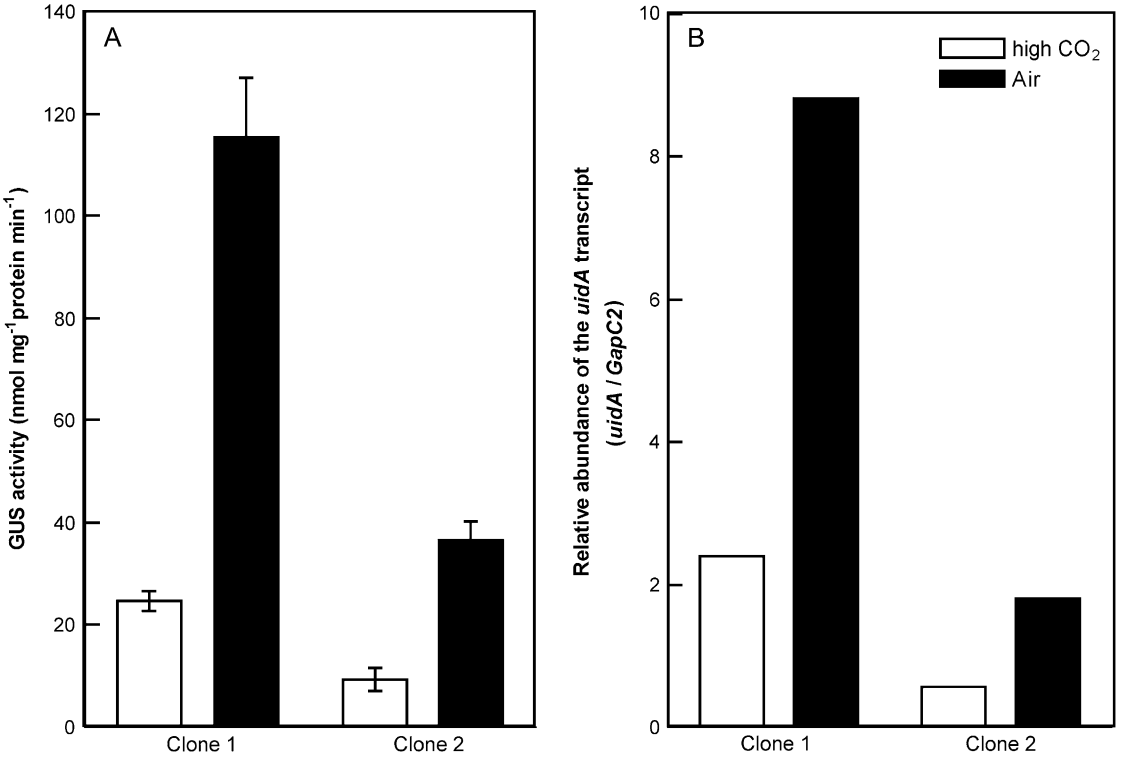

Figure 2. Levels of GUS activities and the GUS gene (*uidA*) transcript in two independent clones of transformants containing the *uidA* reporter. A, Levels of GUS activity in lysates of cells grown in high CO_2 or acclimated to air. Values are means ± SD of three separate experiments. B, Relative levels of the *uidA* transcript in cells grown in high CO_2 and acclimated to air. Transformant cells of *P. tricornutum* grown in 5% CO_2 (white bars) were transferred to air and allowed to acclimate to air for 1 d (black bars) at 20°C under continuous illumination. GUS activity was measured as described in the text. Real-time PCR was carried out with the Smart Cycler thermal cycler system (Cepheid). Relative amount of the *uidA* product was quantified as ratios to levels of the *GapC2* product as an internal standard.

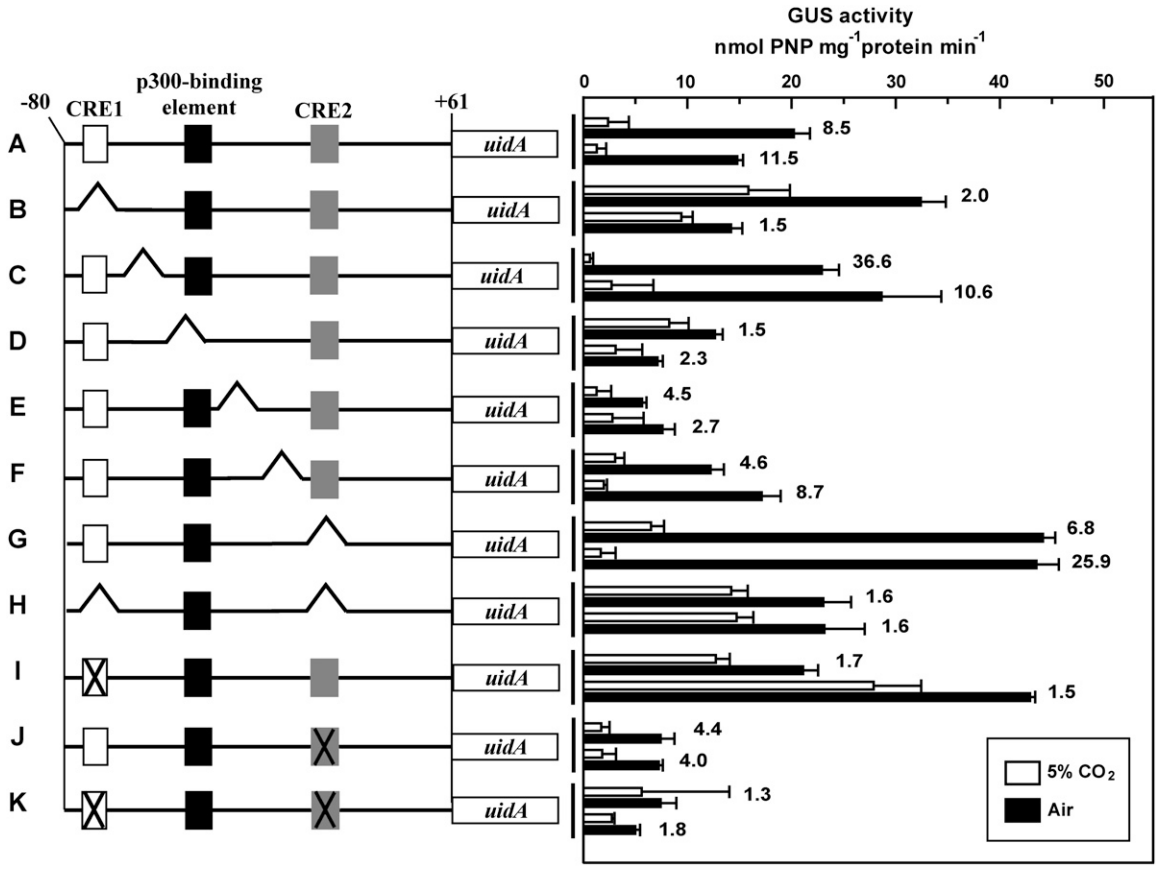

Figure 3. Activities of the intact and manipulated core regulatory regions of the *ptca1* promoter, which were reported by GUS expression levels. Constructs A to K are depicted in the left half and GUS activities in transformants with each construct are shown in the right half. Two independent clones of each transformation were subjected to the GUS assay. Three putative cis-elements, CRE1, p300-binding element, and CRE2, are indicated by white, black, and gray boxes, respectively. A, The intact P*ptca1* core regulatory region with 61 bp of the 5′-untranslated region, which is followed by the *uidA* reporter gene. B to G, A series of 10-bp deletions was carried out to construct A at −71 to −62, −61 to −52, −51 to −42, −41 to −32, −32 to −23, and −22 to −13 bp relative to the transcription start site. H, Regions including both CRE1 (−71 to −62) and 2 (−22 to −13) were deleted. I to K, Either or both CRE1 and 2 were substituted by their respective antisense sequences. Deleted and substituted portions are indicated as broken lines and cross marks, respectively. Values are means ± SD of three separate experiments. CO_2-grown cells (white bars) were transferred to air and were acclimated to air in the light for 1 d (black bars). Values are means ± SD of three separate experiments. GUS activity ratios (air/5% CO_2) are shown to the right of bars.

levels of *uidA* in air, whereas the antisense substitution of CRE2 did not (Fig. 3, G and J). Deletions of sequences located between CREs and the p300-binding element did not cause significant reduction of CO_2 sensitivity of P*ptca1* (Fig. 3, C, E, and F).

P*ptca1* Activities Driven with Substituted Human Cytomegalovirus Minimal Promoter

We further followed up the function of P*ptca1* with a substituted minimal promoter. The region, −10 to +61 relative to the transcription start site of P*ptca1*, containing the initiator-like sequence (CACA) at −2 to +2 was substituted with the human cytomegalovirus (CMV) minimal promoter (mPCMV), which is a TATA-type promoter (Fig. 4, left). The CO_2 responses of the core regulatory region of P*ptca1* (−80 to −11) were well conserved (Fig. 4L) and were not changed significantly by deleting or substituting CRE2 (Fig. 4, O and R). Both deletion and/or antisense substitution of CRE1 and p300-binding element again revealed the apparent tendency of *uidA* derepression under 5% CO_2, and *uidA* expression became constitutive by losing response to CO_2 (Fig. 4, M, N, P, Q, and S). mPCMV alone did not show any CO_2 response and was highly constitutive (Fig. 4T).

Responses of P*ptca1* to the cAMP Analog and the cAMP Phosphodiesterase Inhibitor

To clarify whether cAMP is involved in the CO_2-responsive regulation of *ptca1* expression, responses to an introduced P*ptca1* core regulatory region, with or without CRE1, were analyzed using *uidA* as the

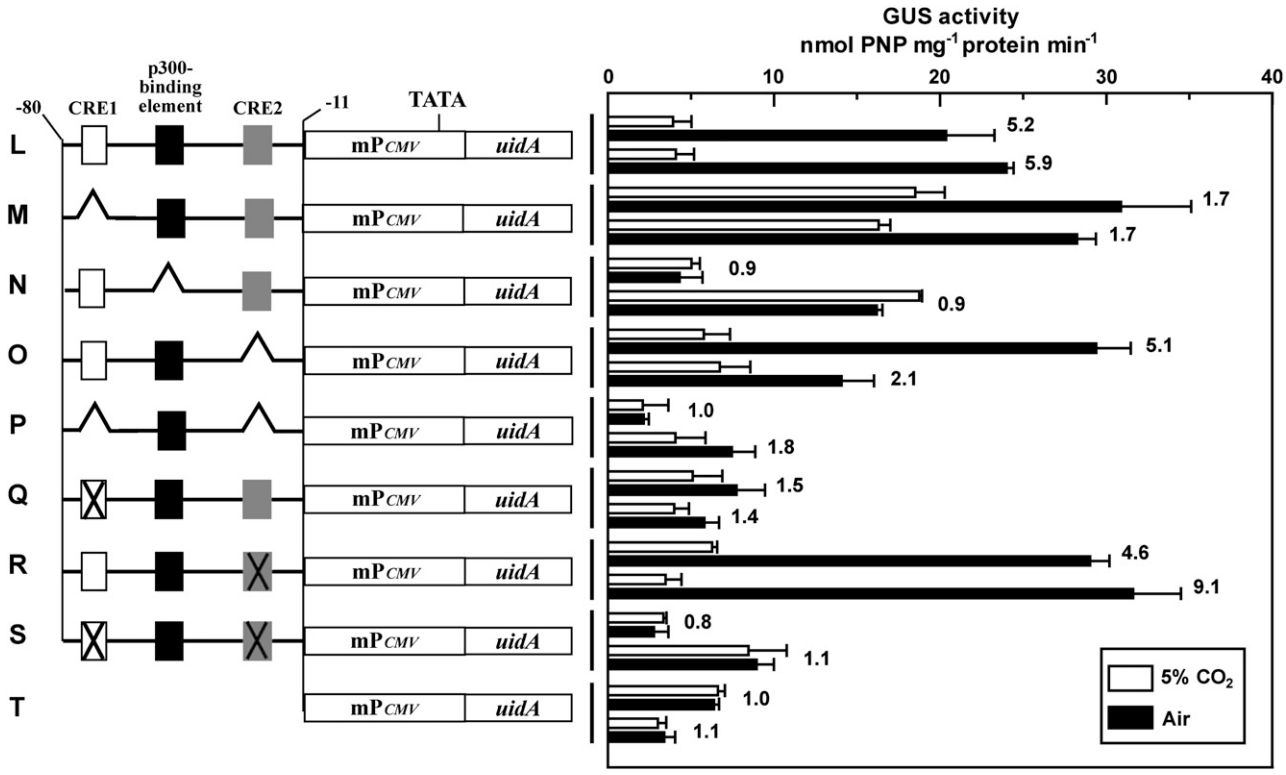

Figure 4. Activities of the intact and manipulated core regulatory regions of P*ptca1*, which are driven by mPCMV. The putative minimal-promoter sequence of P*ptca1* and the 5′-untranslated region (−11 to +61), which includes the putative initiator region, was substituted by the minimal-promoter region of CMV. The left and the right halves indicate constructs and GUS activities in each transformant, respectively. L to S, Substituted forms of the constructs A, B, D, G, H, I, J, and K in Figure 3; T, *uidA* ligated to mPCMV alone. The 5% CO_2-grown cells (white bars) were transferred to air and were acclimated to air in the light for 1 d (black bars). Deleted and substituted portions are indicated as broken lines and cross marks, respectively. Values are means ± SD of three separate experiments. GUS activity ratios (air/5% CO_2) are shown to the right of bars.

reporter during acclimation of *P. tricornutum* cells from 5% CO_2 to air, in the presence of 0.5 and 1.0 mM of the cAMP analog dibutyryl cAMP (dbcAMP) or the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). Interestingly, in the transformant with a complete P*ptca1* core regulatory region, induction of GUS activity during acclimation to air was largely abolished by treatment of *P. tricornutum* cells with these drugs in a dose-dependent manner. That is, GUS activity was 18% and 5% of that in untreated air-acclimated cells in the presence of 0.5 and 1.0 mM dbcAMP, respectively, and, similarly, 28% or 12% in the presence of 0.5 and 1.0 mM IBMX, respectively (Fig. 5, top). In sharp contrast, *uidA* expression was insensitive to the strong repressive effects of these two drugs in the transformant with deleted CRE1, but was almost fully derepressed in air-acclimated cells under these drug treatments or in 5% CO_2-grown cells without drugs (Fig. 5, middle). The same held true when CRE1-deleted P*ptca1* was driven by mPCMV (Fig. 5, bottom).

To examine the direct toxicity of dbcAMP and IBMX to *P. tricornutum* cells, 5% CO_2-grown cells were transferred to air in the presence of both 1.0 mM dbcAMP and 0.5 mM IBMX, and these drugs were removed from the bulk medium after 6 h of acclimation to air. GUS expression was quickly derepressed upon removal of these drugs and increased for the next 12 h (Fig. 6, squares). The time course of the derepression was similar to that in air-acclimating cells without the drug treatment (Fig. 6, black circles and squares), whereas cells maintained under these drugs did not express GUS (Fig. 6, white circles).

Repression of the Endogenous *ptca1* by dbcAMP and/or IBMX in Air

Responses of accumulations of the endogenous *ptca1* transcript to the treatment with dbcAMP and/or IBMX were examined during acclimation of 5% CO_2-grown cells to air (Fig. 7). As described by Harada and Matsuda (2005) previously, the *ptca1* transcript was trace when grown in 5% CO_2, whereas it accumulated about 40-fold of that in 5% CO_2-grown cells after acclimation to air for 2 d (Fig. 7). Levels of the *ptca1* transcript were found to be about 13%, 11%, and

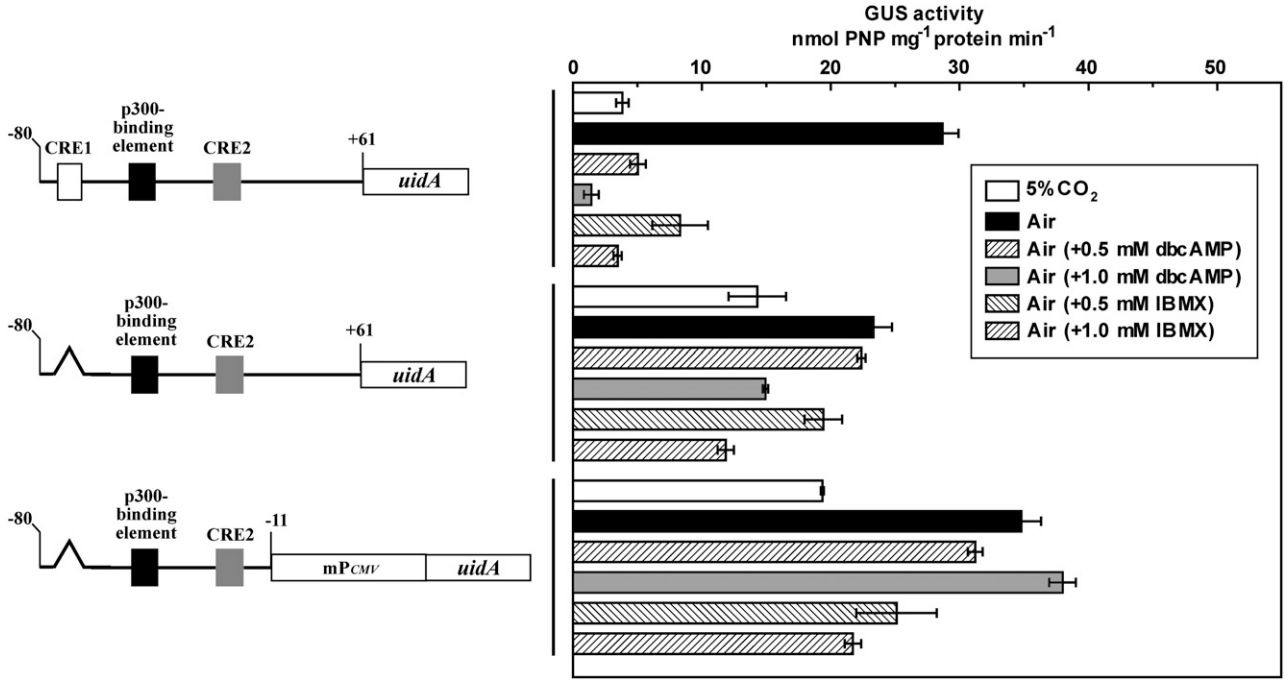

Figure 5. Activities of the CRE1-deleted P*ptca1* during acclimation from 5% CO_2 to air under illumination in the presence or the absence of dbcAMP or IBMX. The left and the right halves indicate constructs and GUS activities, respectively. Two different concentrations of dbcAMP and IBMX were used. Top, A transformant with construct A; middle, a transformant with construct B; bottom, a transformant with construct M. The 5% CO_2-grown cells (white bars) were transferred to air and allowed to acclimate to air for 1 d in the absence (black bars) or the presence of drugs (striped and gray bars). Deleted portions are indicated as broken lines. Types of drug treatment are indicated in the inset. Values are means ± SD of three separate experiments.

4% of those in air-acclimated cells when 0.5, 1.0, and 10 mM dbcAMP, respectively, were added to the medium during acclimation to air (Fig. 7). Similarly, accumulation of the *ptca1* transcript dropped to below 3% of that in the air-acclimated cells when 0.5 and 1.0 mM IBMX were added during acclimation to air (Fig. 7). The transcript level of the endogenous *ptca1* was also suppressed to about 5% of that in air-acclimated cells during acclimation to air under the treatment with both 1.0 mM dbcAMP and 0.5 mM IBMX for 6 h, but

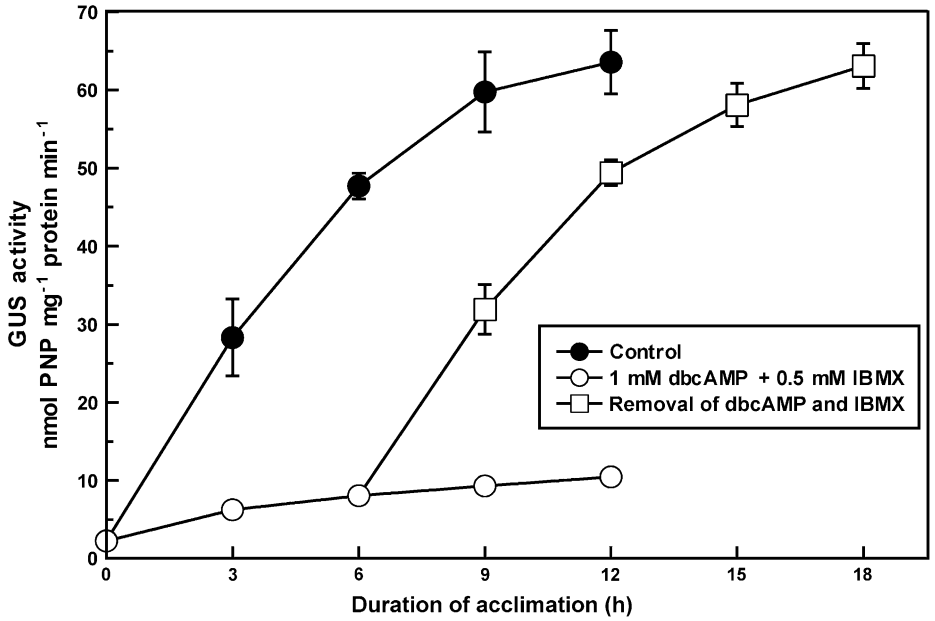

Figure 6. Recovery of GUS reporter expression after the removal of dbcAMP and IBMX in air. The 5% CO_2-grown cells of *P. tricornutum* transformant containing the reporter construct A (Fig. 3A) were transferred to air in the presence (white circles) or the absence (black circles) of 1.0 mM dbcAMP and 0.5 mM IBMX. Cells were allowed to acclimate to air for 12 h, and GUS activity was measured at every 3 h after starting the acclimation. A part of air-acclimating cells under the drug treatment was washed at 6 h of acclimation with CO_2-free F/2ASW to remove dbcAMP and IBMX from the medium and was allowed to acclimate to air for the next 12 h (white squares). GUS activity was measured as described in the text. Acclimation was carried out at 20°C under the photon-flux density of 50 μmol m^{-2} s^{-1}. Values are means ± SD of three separate experiments.

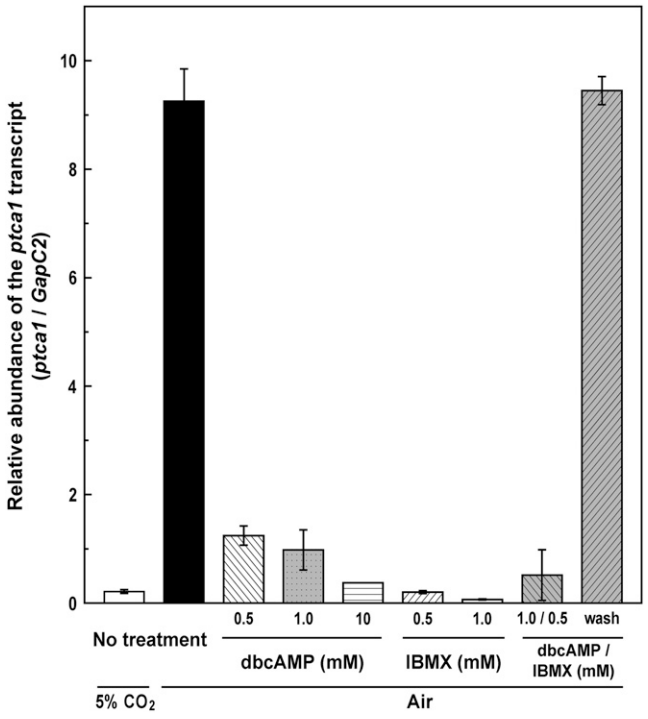

Figure 7. Effects of dbcAMP and IBMX treatments on transcript levels of the endogenous *ptca1* during acclimation from 5% CO_2 to air. Wild-type cells of *P. tricornutum* grown in 5% CO_2 were transferred to air and allowed to acclimate to air for 2 d at 20°C in the absence or presence of 0.5, 1.0, or 10 mM dbcAMP and/or 0.5 or 1.0 mM IBMX as indicated in the diagram. In a part of the experiment, an aliquot of air-acclimating cells under the treatment with both 1.0 mM dbcAMP and 0.5 mM IBMX for 6 h (1.0/0.5) was washed with F/2ASW and allowed to acclimate to air for the next 12 h (wash). Total RNA was extracted from each cell and relative amounts of the *ptca1* product were quantified as ratios to levels of the *GapC2* product as an internal standard using the quantitative real-time PCR technique. Values are means ± SD of three separate experiments.

increased to a level that was equivalent to that in air-acclimated cells within 12 h in air after removal of these two drugs from the bulk medium (Fig. 7).

Responses of P*ptca1* to Light

We previously showed that about 60% of PtCA1 expression in air condition was repressed in the absence of light (Harada et al., 2005). The possibility of participation of a cAMP-related element to P*ptca1* regulation in response to light was examined using *uidA*-reporter constructs with and without CRE1 (Fig. 8, left). The P*ptca1* core regulatory region with added mPCMV showed a clear repression to about 43% the maximum air-level *uidA* expression in the dark (Fig. 8, top), but repression was totally lost by deleting CRE1, irrespective of the type of minimal promoter (Fig. 8, middle and bottom). It was also noted that high-CO_2 condition in the light always showed slightly stronger repressive effect on P*ptca1* than that in air in the dark, irrespective of the existence of CRE1 and of types of minimal-promoter sequence (Fig. 8).

DISCUSSION

The promoter region of *ptca1* was previously isolated and it was demonstrated that the critical CO_2-response sequence was located downstream −70 bp relative to the transcription start site (Harada et al., 2005). This relatively short core regulatory sequence comprises two putative cAMP-response elements, CRE1 at −70 to −63 and CRE2 at −21 to −14, and a putative p300-binding element at −52 to −46 relative to the transcription start site (Harada et al., 2005; Fig. 1). A rough truncation experiment, which was previously done on this region, suggested that these elements would participate in repressive regulation of the *ptca1* expression in response to increase in [CO_2] (Harada et al., 2005). A precisely targeted deletion or an antisense substitution of CRE1, conducted in this study, was clearly related to derepressions of P*ptca1* under 5% CO_2 condition (Figs. 3, B and I, and 4, M and Q). Although GUS expression levels varied depending on clones presumably due to the position effects on inserted fragments, GUS expression ratios were found to be relatively stable and revealed characteristics of each manipulated construct of P*ptca1*. This apparent tendency of the P*ptca1* derepression in high CO_2 was also held unchanged in the case of double deletions or antisense substitutions of both CRE1 and CRE2 (Figs. 3, H and K, and 4, P and S). CRE2 is, however, unlikely to be relevant to the repressive activity of the P*ptca1* core regulatory region under high-CO_2 conditions, since a single deletion or substitution of the CRE2 region did not cause a reduction in the CO_2 sensitivity of *uidA* expression (Fig. 3, G and J). It is also likely that the p300-binding region would participate significantly in the CO_2 responses of P*ptca1* (Figs. 3D and 4N). These data clearly indicate that the CO_2 response of the P*ptca1* core regulatory region operates irrespective of the structures of the minimal-promoter region and that CRE1 and the p300-binding element are required for the repression of *ptca1* in response to increases in the ambient [CO_2].

In this study, mPCMV was shown to operate in cells of the marine diatom *P. tricornutum*. GUS expression driven by mPCMV alone without P*ptca1* was highly constitutive and did not show any CO_2 response (Fig. 4T). The activity of mPCMV did not disturb the CO_2-responsive regulatory function of P*ptca1* (−80 to −11). These data clearly indicate that CO_2 response of P*ptca1* is not governed by the sequence between −10 and +61 relative to the transcription start site. It should be also noted that a mammalian viral promoter could be used as a molecular tool for marine diatoms.

The occurrences of CREs and the p300-binding element on the CO_2-responsive promoter are the striking features that indicate the participation of cAMP in the CO_2 response of marine diatoms. Our preliminary

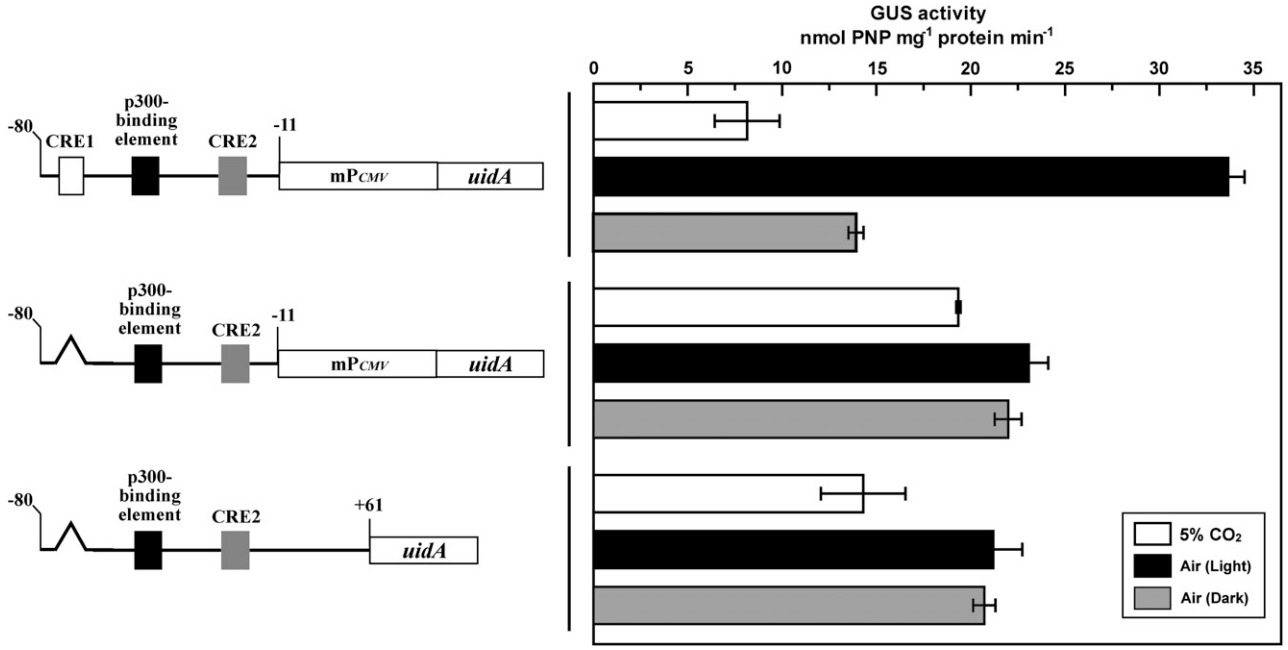

Figure 8. Activities of the intact or the CRE1-deleted P*ptca1* during acclimation from 5% CO_2 to air under illumination or in the dark. The left and the right halves indicate constructs and GUS activities, respectively. Top, A transformant with the construct L shown in Figure 4; middle, a transformant with the construct M shown in Figure 4; bottom, a transformant with the construct B shown in Figure 3. The 5% CO_2-grown cells (white bars) were transferred to air and were allowed to acclimate to air in the light (black bars) or the dark (gray bars) for 1 d. Deleted portions are indicated as broken lines. Values are means ± SD of three separate experiments.

attempts, however, failed to detect significant changes in cAMP concentration in response to growth [CO_2] in a cell lysate of *P. tricornutum*. Cyclic AMP is a highly redundant molecule that operates in many different signal transduction pathways as a second messenger, and, therefore, concentration changes in cAMP in a particular physiological response are not precisely detected in a whole-cell lysate. This consideration prompted us to quantify relative changes in expression levels of the GUS reporter gene, which is driven with manipulated P*ptca1*, under treatments with modulator reagents for levels and activities of the cytosolic cAMP. The results obtained in this study clearly showed that either supply of the cAMP analog dbcAMP or an enrichment of the steady-state level of cAMP by inhibiting cAMP degradation with IBMX strongly abolished GUS expression in the transformant with a complete P*ptca1* core regulatory region during acclimation to air (Fig. 5). These repressive effects by high CO_2 or by these two drugs on P*ptca1* largely disappeared in the absence of CRE1, and *uidA* was derepressed under these treatments (Fig. 5). Furthermore, dbcAMP and IBMX showed little toxicity to cells of *P. tricornutum* (Figs. 6 and 7). It is thus likely that cAMP is the key component in transmitting the high-CO_2 signal to repress P*ptca1* via the function of CRE1 in *P. tricornutum*. It is also noteworthy that increases in the dose of dbcAMP and IBMX from 0.5 to 1.0 mM exhibited trace repressive effects on the CRE1-deleted P*ptca1* (Fig. 5). This probably indicates that CRE1 alone is not sufficient to account for the repressive effects of dbcAMP and IBMX but requires additional elements, such as the p300-binding element, for full function. It is also confirmed that dbcAMP and IBMX strongly repressed the transcription of the endogenous *ptca1* even under air-level CO_2 condition (Fig. 7), indicating clearly that increase in the cytosolic cAMP level plays a key role in repression of the native *ptca1* promoter.

Responses of CCM components to light in eukaryotic algae are also diverse, but the absence of light is strongly or moderately repressive in many algal species (Matsuda and Colman, 1995; Villand et al., 1997; Kucho et al., 1999; Bozzo and Colman, 2000; Harada et al., 2005). However, the light signaling pathway in CCM regulation has not been elucidated in detail. In the marine diatom *P. tricornutum*, the *ptca1* gene is repressed in the dark to about 40% of that under illumination (Harada et al., 2005), but the promoter sequence of *ptca1* is not similar to any of known CO_2-responsive promoters (Villand et al., 1997; Kucho et al., 1999; Harada et al., 2005). This indicates that the origins of CO_2-response systems are also diverse. The P*ptca1* core regulatory region with added mPCMV showed a clear repressive effect on *uidA* expression in the dark (Fig. 8, top), but the repression was totally lost by deleting CRE1 irrespective of the type of minimal promoter (Fig. 8, middle and bottom). It is strongly suggested from these results that cAMP pathways

transmit both CO_2 and light signals via CRE1. Derepressions of *uidA* expression by deleting CRE1 were almost 100% and GUS levels reached the maximum in these transformants grown in the dark (Fig. 8, middle and bottom), whereas trace repressions of *uidA* were always observed in high CO_2 in the light in these CRE1-deleted transformants (Fig. 8, middle and bottom). It is probable from these results that the repression of P*ptca1* in the absence of light is governed primarily by CRE1 but no other cis-element would be required, whereas the CO_2 response, as described above, would require cooperation of other regulatory elements presumably related to cAMP. This consideration in turn makes us question whether light and CO_2 signals might be transmitted to P*ptca1* in different ways via transcription factors that interact either primarily with CRE1 or cooperatively with both CRE1 and other regulatory elements.

In animal cells, CRE has been reported as a cis-element that is targeted by the CRE-binding protein, CREB, whose phosphorylated form binds to the CREB-binding protein, CBP (Chrivia et al., 1993), and stimulates the formation of a basal transcriptional complex (Kwok et al., 1994). This process operates typically in transcriptional activations in response to hormones, neurotransmitters, and olfactants. The p300 is known as a functional homolog of CBP and also activates transcriptional processes in mammalian development by interacting with CREB (Rikitake and Moran, 1992; Tanaka et al., 1997). All of these animal systems, mediated by cAMP and CRE-related factors, function to activate transcriptions. In contrast, examples of repressive regulation of cAMP-mediated gene expression are limited. One of the best known cases is the catabolite-repression model in yeast, in which exposures to some favorable organic carbon sources, such as Glc, cause increases in cytosolic cAMP level and in turn repress genes controlling bioenergetic pathways, such as alcohol dehydrogenase and Fru-1,6-bisphosphatase (Cherry et al., 1989; Zaragoza et al., 1999). CCM repression in diatoms appears to be homologous to the catabolite-repression model as it is a down-regulation model by excess substrate for photosynthesis. However, catabolite repression in yeast has not been related to CRE or p300-binding element. Repression of P*ptca1* is thus a new repression model by cAMP via a CRE-related promoter sequence.

Molecular research on acclimation of marine diatoms to changes in environmental factors is extremely important considering its ecophysiological significance, although it is still at a rudimentary stage. Responses of marine diatoms to changes in physicochemical factors, such as fluid motion, osmotic stress, iron, light, and [CO_2], might be controlled by specific receptors and feedback mechanisms probably mediated, at the cytosolic level, by second messengers, such as Ca^{2+} (Falciatore et al., 2000) and cAMP (this study). It is also suggested in this study that there might be a fine discrimination of the CO_2 signal from the light signal at the promoter level in a marine diatom. CO_2 input at the ocean surface may thus act as a direct signal to the marine ecosystem as one of possible regulatory factors of primary productivity.

MATERIALS AND METHODS

Cells and Culture Conditions

The marine diatom *Phaeodactylum tricornutum* Bolin (UTEX 642) was obtained from the University of Texas Culture Collection and was grown in artificial seawater, which was supplemented with half-strength Guillard's "f" solution (F/2ASW; Guillard and Ryther, 1962; Harrison et al., 1980) under continuous illumination (50 μmol m^{-2} s^{-1}) at 20°C with constant aeration with 5% CO_2 or ambient air. Fifty milliliters of 5% CO_2-grown cells were centrifuged at 3,500*g* at 20°C for 5 min, washed twice with 10 mL of CO_2-free F/2ASW, resuspended in 50 mL of CO_2-free F/2ASW, and allowed to acclimate to air by aeration with ambient air for 1 to 2 d. In some experiments, 0.5, 1.0, or 10 mM dbcAMP and/or IBMX were added during the acclimation to air. Prior to the acclimation to air in the dark, 5% CO_2-grown cells were dark adapted for 1 h in 5% CO_2.

Preparation of Chimeric Constructs

The upstream region of the *ptca1* open reading frame was isolated as described previously (Harada et al., 2005). Using this region as a template, the core regulatory region, −80 to +61 bp relative to the transcription start site, was amplified by PCR. The core regulatory region was phosphorylated and inserted into the blunt-ended site of transformation vector pFcpApGUS (Harada et al., 2005; Fig. 3A). To create deletion constructs (Fig. 3, B–H), PCR primers were designed to perform an inverse elongation from the sequence neighboring each deletion site in the P*ptca1* (Fig. 3, B–H) and PCR was done using construct A as a template. A double-deletion construct (Fig. 3H) was created in the same way as above using construct B as a template. To create constructs I to K (Fig. 3), PCR primers that contain antisense substitution at the 5′ termini were designed and amplified by PCR using construct A as a template. A double-substitution construct (Fig. 3K) was created by PCR on construct I as a template. To create constructs L to S (Fig. 4), the deleted or the substituted constructs of P*ptca1* (−80 to −11), which lack the region containing the initiator and the omega sequence (−10 to +61), were amplified on templates of constructs A to K (Fig. 3) and fused with mPCMV by PCR. These fragments were then phosphorylated and inserted into the blunt-ended site of pFcpApGUS. As a control, *uidA* was fused with mPCMV alone and designated as construct T (Fig. 4).

Transformation of *P. tricornutum*

Transformation was carried out as described by Zaslavskaia et al. (2000). *P. tricornutum* cells grown in 5% CO_2 under continuous illumination were harvested at the mid-logarithmic phase (OD_{730} = 0.3–0.4). Approximately 5×10^7 cells were spotted as a plaque of 2.5-cm diameter on the surface of the F/2ASW agar plate. Five hundred micrograms of tungsten microcarriers (1.1 μm particle size) were coated with approximately 1.0 μg of plasmid DNA containing 1.0 M $CaCl_2$ and 16 mM spermidine. PDS-1000/He biolistic particle-delivery system (Bio-Rad) was used for microprojectile bombardments of microcarriers. The bombardment was done at 1,550 psi to the cells in the chamber under the pressure of −27 inches mercury with a target distance of 6 cm. Bombarded cells were cultured for 1 d under illumination and were suspended in 5 mL of F/2ASW. After the centrifugation at 3,000 rpm at 20°C for 5 min, cells were resuspended in 0.3 mL of F/2ASW, plated onto F/2ASW agar plate containing 100 μg mL^{-1} Zeocin.

GUS Assays

The transformants were cultured in 100 mL of F/2ASW in 5% CO_2 or air. Five to 10 mL of cells were harvested and disrupted by a sonicator (Ultrasonic disruptor model UD-201; TOMY Seiko) at output level 3 in 0.5 mL of GUS extraction buffer [50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100], and then centrifuged at 13,000 rpm for 5 min at 4°C. Twenty microliters of lysate was added to 980 μL of GUS assay buffer (10 mM *p*-nitrophenyl β-D-glucuronide in

the GUS extraction buffer) and incubated at 37°C for 1 h. The reaction was terminated by the addition of 400 μL of 0.5 M Na_2CO_3 every 10 min after starting the reaction and the optical density of *p*-nitrophenol released from the substrate was measured at 405 nm.

Quantitative Real-Time PCR

Total RNA was extracted from 5% CO_2-grown cells, air-acclimated cells, or cells treated with dbcAMP and/or IBMX during acclimation to air using RNeasy Plant Mini kit (QIAGEN) according to the protocol provided by the manufacturer. Total RNA (1 μg) was reverse transcribed using oligo$(dT)_{20}$ primer (TOYOBO) and reverse transcriptase (Revertra Ace; TOYOBO) to form single-stranded cDNA. To amplify the *uidA* (accession no. S69414) and the *ptca1* (accession no. AF414191) cDNAs, sets of forward and reverse primers, GUSRTF (5′-TTGCCAACGAACCGGATA-3′) and GUSRTR (5′-AATCGCCGCTTTGGACATAC-3′), and CA1F (5′-TCACAATTCCTAGCAGAAAATCATCG-3′) and CA1R (5′-ACGCATCCAATGTACAAGTACTTGGG-3′), were used for PCR. Quantitative PCR was standardized on known amounts of template using plasmids containing the *uidA* or the *ptca1* cDNA. To normalize the quantification, the levels of the transcript of the cytosolic glyceraldehyde-3-P dehydrogenase gene (*GapC2*; accession no. AF063805), which is constitutively expressed under both 5% CO_2 and air, were measured as an internal marker. Amplification of the *GapC2* cDNA was done using the GapC2F (5′-TTTTTCGCCTTTCTAAACATCAGTT-3′) and GapC2R (5′-TACTCGGGCGTCAAGAAGG-3′) primer pair. The quantitative PCR was carried out with a Smart Cycler thermal cycler system (Cepheid) and Takara Ex Taq R-PCR Version 2.0 (Takara Bio) or SYBR Premix Ex Taq (Takara Bio) under PCR conditions as follows: for *uidA*, heating at 95°C for 10 s, followed by 45 cycles of denaturing at 95°C for 5 s, annealing at 64°C for 20 s, and extension at 72°C for 10 s; and for *ptca1* and *GapC2*, same conditions, except annealing at temperatures of 63°C and 60°C, respectively.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers S69414, AF414191, AF063805, and M64944.

ACKNOWLEDGMENTS

We thank Dr. Brian Colman (York University, Toronto) for critical reading and comments on this manuscript, Ms. Nobuko Higashiuchi for her technical assistance, and Ms. Miyabi Inoue for her skillful secretarial aid.

Received July 11, 2006; accepted September 21, 2006; published September 29, 2006.

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