Cloning and Characterization of High-CO₂-Specific cDNAs from a Marine Microalga, *Chlorococcum littorale*, and Effect of CO₂ Concentration and Iron Deficiency on the Gene Expression

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Two cDNA clones exclusively induced under an extremely high-CO₂ concentration (20%) were isolated from *Chlorococcum littorale* by differential screening and named HCR (high-CO₂ response) 1 and 2, respectively. The amino acid sequence of the protein encoded by HCR2 exhibited homology to the gp91-phox protein, a critical component of a human phagocyte oxidoreductase, and to the yeast ferric reductases, *Saccharomyces cerevisiae* FRE1 and FRE2 and *Schizosaccharomyces pombe* Fpr1. The induction of both HCR mRNAs required extremely high-CO₂ conditions and iron deficiency, being suppressed under air conditions and by iron sufficiency, suggesting that the expression of these two HCR genes required extremely high-CO₂ conditions and iron deficiency in combination. The HCR2 protein was detected in the membrane fractions of cells grown under conditions which would favor the induction of HCR2-mRNA and the protein level was lowered when the cells were transferred from iron deficient to 10 μM FeSO₄ conditions (with 20% CO₂).

Key words: *Chlorococcum littorale* — CO₂ concentration — Differential screening — Iron stress.

Many microalgae become acclimatized to various types of environmental stress. When microalgae are grown in air (limited-CO₂ conditions), carbonic anhydrase and an active transport system for inorganic carbon (both for the CO₂-concentrating mechanism, ccm) are induced and, as a result, the affinity for inorganic carbon in photosynthesis is markedly increased. On the other hand, ccm was suppressed when these cells were grown in air enriched with 1-5% CO₂ (Aizawa and Miyachi 1986, Badger and Price 1992). The microalgal cells grown in air and in 1-5% CO₂-enriched air are generally referred to as "low-CO₂" and "high-CO₂" cells, respectively.

It has been well documented that extremely high concentrations of CO₂ suppressed the rate of photosynthesis and the growth of microalgae (Osterlind 1950, Nielsen 1955, Lee and Tay 1991). Some species of microalgae have recently been found to grow under extremely high-CO₂ conditions. A new species of marine green alga, *Chlorococcum littorale* (Chihara et al. 1994) was shown to grow under high-CO₂ conditions up to 60% (Kodama et al. 1993). This finding led us to the study of "extremely high-CO₂" cells. Pesheva et al. (1994) have reported that the lag period in the growth observed in the course of adaptation from air to 40% CO₂ was due to a temporal decrease in photosystem II activity induced by such an extremely high-CO₂ concentration. Iwasaki et al. (1996) confirmed this by a quenching analysis of chlorophyll fluorescence. However, there are still many questions on the influence of "extremely high-CO₂ concentration" on the growth and physiological processes of microalgae.

To investigate the relationship between extremely high-CO₂ (20%) stress and gene expression, we cloned two independent cDNAs from *C. littorale* cells which were induced under such conditions. An analysis of the sequence homology, the expression of HCR (high-CO₂-response) mRNAs indicated that the expression was controlled not only by an extremely high-CO₂ concentration, but also by iron deficiency.

Materials and Methods

**Algal cells and culture**—*Chlorococcum littorale* cells were grown in an MC medium (1.5% NaCl added to a fresh water-based medium or 1.2% NaCl in a 10% sea water-based medium, containing 10 μM FeSO₄ under continuous illumination (250 μmol m⁻² s⁻¹) and aeration with ordinary air at 25°C in a 5-liter culture vessel (Kodama et al. 1993). The air-grown cells were harvested by centrifugation (1,600 × g for 5 min), resuspended in the fresh medium that had been supplemented with various concentrations of an FeSO₄ solution immediately before use, and then aerated with ordinary air or air enriched with 20% CO₂. The pH value of the medium was controlled to 5.5 with 1 M H₂SO₄ or 1 M NaOH. The packed cell volume (PCV) was determined by centrifuging the cells of *Chlorococcum littorale* in hematocrit tubes for 10 min at 1,600 × g, centrifugation being repeated until a constant value was obtained.

**Isolation of RNA**—The cells were harvested by centrifugation (1,600 × g for 5 min), and the resulting pellet was transferred to liquid nitrogen. The frozen pellet was powdered with a mortar and pestle, and then lysed with a denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5%
sarcosyl and 0.1 M 2-mercaptoethanol). Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method of Chomczynski and Sacchi (1987) and precipitated for 2 to 14 h at 4°C after adding 10 M LiCl to a final concentration of 2 M into 1 mg ml⁻¹ of RNA solution. Poly(A)⁺ RNAs were prepared by using Oligotex-dT30 Super equipment (TaKaRa, Kyoto, Japan).

Preparation and differential screening of the cDNA library—Double-stranded cDNAs were synthesized from poly(A)⁺ RNAs that had been prepared from Chlorella vulgaris cells grown under 20% CO₂ for 2 days (20% CO₂ cDNA) or ordinary air for 4 days which exhibited the same cell density as a 2-day culture under 20% CO₂ conditions. The cDNA library was constructed by using the agar10 vector from 20% CO₂ cDNA and contained 4.3 x 10⁶ pfu μg⁻¹ cDNA. The plaque hybridization method (Sambrook et al. 1989) was used to differentially screen about 40,000 plaques of the primary cDNA library. The plaques that hybridized more strongly with the probes of 20% CO₂ cDNA were selected.

Subcloning and DNA sequencing of the cDNA inserts—A DNA fragment was subcloned into the pUC19 vector (TaKaRa), DNA fragments of 1.4 kbp (HCR1) and 3.1 kbp (HCR2) digested by BamHI being used for subcloning. To obtain the 5'-end of HCR2 cDNA, the upstream sequence was examined with a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, U.S.A.). The nucleotide sequence of this cDNA was determined by the method of Sanger et al. (1977) with a PRISM Dye Terminator cycle sequencing kit (model 373A; Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide and amino acid sequence analyses were conducted by GENETYX software (Software Development Co., Tokyo, Japan).

Northern hybridization—Air-grown cells were exposed to 20% CO₂ conditions, and then harvested at the indicated times to isolate total RNA. Northern hybridization was carried out as described by Sambrook et al. (1989). Ten μg of RNA was separated on formaldehyde/agarose gel, blotted onto a Hybond-N⁺ membrane (Amersham, Buckinghamshire, England) and hybridized with cDNA fragments of HCR1 and 2 labeled by random priming with [³²P]dCTP (Amersham) using a kit from TaKaRa. The intensity of each signal was detected with a Bio-imaging analyzer (BAS1000; Fuji Film, Japan).

Cell fractionation—Cells were harvested by centrifugation (1,600 x g for 5 min) and resuspended in 1 ml of a homogenizing buffer (10 mM Tris-Cl [pH 7.5], 10 mM EDTA, 5 mM 6-aminonicotinic acid, 2 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were broken by beadbeater (Biospec Products, Yaski Kikai, Japan) in a 1.5-ml screw-cap tube containing 0.5 mm diameter glass beads while chilling on ice at regular intervals. The resulting suspension was centrifuged (1,600 x g for 2 min) to remove unbroken cells, the resulting supernatant being ultracentrifuged (164,000 x g for 30 min) to precipitate the crude membrane fractions. The resulting membrane pellets were resuspended in the homogenizing buffer, and the supernatant was used as the soluble fraction.

Preparation of antibodies—The HCR2 clones were digested by HindIII, and the 550 bp fragment was subcloned into the pET28b vector (Novagen). The HindIII fragment of protein expressed in E. coli BL21(DE3) was purified and used as the protein antigen. The antibody was produced in a New Zealand white rabbit (obtained from Sawady Technology, Japan).

SDS-PAGE and immunoblotting—SDS-PAGE in 10% polyacrylamide gel was carried out by the method of Laemmli (1987). The membrane proteins were precipitated by acetone (75% final concentration) and heated in a solution of 50 mM Tris-Cl at pH 6.8, 2% SDS, and 1% 2-mercaptoethanol at 70°C for 10 min, before being separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) filter (Bio-Rad) by the protocol of Towbin et al. (1979). The membrane sheet was blocked with 5% skim milk and then washed with Tris-saline containing 0.1% Tween-20. The antigen against the HindIII fragment of HCR2 was used as the primary antibody, the binding antibody signal being amplified with protein-A gold and enhanced by a Gold enhancement kit (Bio-Rad).

Results

Differential screening—To isolate the cDNA clones associated with extremely high-CO₂ conditions from Chlorella vulgaris cells, the differential screening was carried out by hybridization with radiolabeled cDNA probes prepared from 20% CO₂ and air grown cells (see Materials and Methods). Seventy-eight plaques strongly hybridized with [³²P] labeled high-CO₂ cDNA were isolated from a 40,000 plaque cDNA library. The inserted DNA fragment from each λphage clone was analyzed and classified by Southern hybridization, and 78 clones were finally divided into 2 groups, HCR (high-CO₂ response) 1 and 2. HCR1 and 2 respectively contained 54 and 24 clones (Table 1).

DNA sequence analysis of the HCR2-cDNA clone—The amino acid sequences obtained were compared with known sequences in the SWISS PLOT database. Although no sequence homology was found for HCR1, the deduced HCR2 protein had 815 amino acid residues with a predicted molecular weight of 90.8 kDa. Fig. 1 shows the nucleotide and deduced amino acid sequences of HCR2 cDNA. 5'-RACE was carried out to confirm the first part of the coding region of HCR2, which was found to start at nucleotide 44 and to end at nucleotide 2488. A search of the protein database revealed amino acid sequence homology between HCR2, the gp91-phox subunit (Orkin 1989, FRE1 (Dancis et al. 1992), FRE2 (Georgatsou and Alexandraki 1994) and Frp1 (Roman et al. 1993). The identity levels between HCR2 and gp91-phox, FRE1, FRE2 and Frp1 were 22.2% (per 185 amino acid residues), 19.9% (per 307 residues), 18.3% (per 279 residues) and 19.0% (per 126 residues), respectively. While the degree of identity for

Table 1 Characteristics of the high-CO₂-responsive (HCR) cDNA clones isolated by differential screening

<table>
<thead>
<tr>
<th>Clone</th>
<th>Transcript size (kb)</th>
<th>cDNA size (bp)</th>
<th>Number of clones obtained</th>
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<tr>
<td>HCR1</td>
<td>1.5</td>
<td>1,386</td>
<td>54</td>
</tr>
<tr>
<td>HCR2</td>
<td>3.0</td>
<td>3,095</td>
<td>24</td>
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Transcript size was calculated by Northern blots, and cDNA size was obtained from cDNA sequences.
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Each was limited when the full length of each gene was compared, a few clusters of conserved and identical amino acid existed (Fig. 2). Cluster A, which has been proposed to be functioning in FAD binding, was the most well conserved sequence:

ISO CFGIVTFGYVTKFWDL 46

Cluster A, which has been proposed to be functioning in FAD binding, was the most well conserved.

Effect of CO₂ concentration on HCR expression—The induction of the two HCR genes in *C. littorale* cells after transfer from air to 20% CO₂ conditions (see Materials and Methods) was analyzed by Northern hybridization. Air-grown cells were transferred to a fresh medium that had been supplemented with a 10 μM FeSO₄ solution just before inoculation, then ordinary air or air enriched with 20% CO₂ were aerated. When the cells were transferred to 20% CO₂, enhanced growth was found after a lag period which lasted for 1 day (Fig. 3B). mRNAs corresponding to HCR1 and 2 began to accumulate 2 or 3 days after transfer to 20% CO₂ (Fig. 3A), while these mRNAs were not detected in air conditions which lasted for 9 days.

Effect of iron concentration—The cells which had been grown in air and those with the addition of 10 μM FeSO₄ were transferred to a fresh medium containing 0, 10, or 100 μM FeSO₄ as the final concentration and bubbled with 20% CO₂-enriched air. Fig. 4 shows that the induction of both HCR1 and 2 was stimulated by limiting ferrous iron, while it was suppressed by adding 100 μM FeSO₄. In particular, no HCR2 mRNA was detectable, even 4 days after transfer into the 100 μM FeSO₄ medium. The gradual expression of HCR1 that was observed in the presence of 100 μM FeSO₄ might have been due to oxidization of the Fe(II) ion and, hence to a decrease in the soluble iron content in the culture medium by gas bubbling. Although HCR1 and 2 were detected in the presence of 10 μM FeSO₄, both inductions were slower than in the absence of iron. These results suggest that HCR mRNA expression were regulated not only by CO₂ concentration but also by iron concentration. The figure also shows that the expression of HCR1 in response to the change in FeSO₄ concentration was faster than that of HCR2.

Effects of decreasing CO₂ and adding FeSO₄—Fig. 5 (I) shows that HCR mRNAs could be detected 1 to 2 days after transfer from air and under 10 μM FeSO₄ to 20% CO₂ and iron-deficient conditions. When the cells which had been grown with 20% CO₂ and iron deficiency for 2 days were transferred to air and iron-deficient conditions, HCR2 mRNA decreased slowly and became undetectable by the end of day 1 [Fig. 5 (II)]. The suppression of HCR1 mRNA expression was less than that of HCR2. When 10 μM FeSO₄ was added after 2 days of culture with 20% CO₂ in the absence of iron, the expression of both HCR1 and 2 mRNAs was suppressed after 3 hours (day 2.125) [Fig. 5 (III)]. The expression of HCR1 mRNA started again after 6 hours (day 2.25) in spite of the presence of iron. This
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Fig. 2 Comparison of the deduced amino acid sequences of the putative HCR2 protein and gp91-phox, FRE1, FRE2 and Frp1 proteins. Except for HCR2, the amino acid sequences are from the SWISS PLOT database. Shaded residues are homologous to those in the HCR2 protein.

Fig. 3 Effects of high-CO₂ conditions on the induction of the HCR genes (A) and growth (B) in *Chlorococcum littorale*. In the Northern analysis (A), total RNA was prepared after air-grown *C. littorale* cells had been kept in 20% CO₂ or ordinary air for the indicated times (days). Cells were transferred to the culture medium containing 10 μM FeSO₄ at the end of day 0. Ten μg of total RNA prepared from the cells were applied to each lane.

Fig. 4 Immunological detection of the HCR2 protein—To determine how quickly the HCR2 protein would be degraded after transfer from iron-deficient to -sufficient conditions, a Western analysis was carried out. The antiserum raised against the HCR2 polypeptide (see Materials and Methods) was immunoreactive with the about-90 kDa poly-

might also have been the result of oxidization and a decrease of soluble Fe(II) ions as already described.

**Immunological detection of the HCR2 protein**—To determine how quickly the HCR2 protein would be degraded after transfer from iron-deficient to -sufficient conditions, a Western analysis was carried out. The antiserum raised against the HCR2 polypeptide (see Materials and Methods) was immunoreactive with the about-90 kDa poly-

![Amino acid sequence comparison](image)

![Western blot analysis](image)

![Growth graph](image)
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FeSO₄ concentration

<table>
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<th>0 µM</th>
<th>10 µM</th>
<th>100 µM</th>
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<tr>
<td>HCR 1</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>HCR 2</td>
<td>0 1 2 3 4</td>
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Fig. 4 Effects of iron concentration in the growth medium on the induction of HCR mRNAs. Air-grown cells (with 10 µM FeSO₄) were transferred to a medium containing 0, 10 or 100 µM FeSO₄, and air containing 20% CO₂ was bubbled through each medium for the indicated number of days.

peptide from iron-starved cells under 20% CO₂ for 2 days (Fig. 6). The size of the resulting band was similar to the molecular weight (90.8 kDa) predicted from the deduced amino acid sequence of HCR2 (Fig. 1). This immunodetectable band was only found in the membrane fraction, and not in the soluble fraction. Some of the other immunoreactive bands of smaller molecules and smear bands of larger molecules may have been nonspecific, degradation products or aggregates of insoluble HCR2 polypeptides.

The time course of HCR2 polypeptide accumulation during the adaptation process of air-grown cells (in a 10 µM FeSO₄ medium) to the subsequent 20% CO₂ and iron-deficient conditions was thus examined. Membrane proteins from cells collected at different adaptation stages were subjected to an immunoblot analysis. Fig. 7 shows that the 90-kDa HCR2 polypeptide increased 2 days after transfer of cells grown in air with 10 µM FeSO₄ to 20% CO₂ and iron-deficient conditions (left area of Fig. 7A, B). When air was bubbled through these cells, no polypeptide could be detected (right area of Fig. 7A). This polypeptide was also decreased by adding of 10 µM FeSO₄ at the end of the 2nd day (right area of Fig. 7B). These results show good correlation between the immunochemically detected level of polypeptide and the level of corresponding mRNAs (Fig. 5).

Discussion

When air-grown cells of C. littorale were transferred to extremely high-CO₂ conditions, growth enhancement occurred after a period of time, the lag period needed being longer as the concentration of CO₂ became the higher (Pesheva et al. 1994). When low-CO₂ cells were trans-

Fig. 5 Changes in the HCR gene expression in Chlorococcum littorale under various conditions. (I) Air-grown cells with 10 µM FeSO₄ were transferred into an iron-deficient medium (designated as −FeSO₄) at the end of day 0 after being aerated with 20% CO₂ for 3 days. (II) Air-grown cells with 10 µM FeSO₄ were grown under 20% CO₂ for 2 days and then under air for 1 day, both in the absence of FeSO₄ (−FeSO₄). (III) Air-grown cells with 10 µM FeSO₄ were grown in the absence of FeSO₄ (−FeSO₄), and then 10 µM FeSO₄ was added at the end of the 2nd day. Air containing 20% CO₂ was bubbled throughout the experimental period.
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MW 1 2 3 4 5 6
(kD) 94 67 43 30

Fig. 6 Immunoblot analysis of proteins from the total (lanes 1 and 4), soluble (lanes 2 and 5) and crude membrane fractions (lanes 3 and 6) in *Chlorococcum littorale* cells. The cells were grown under 20% CO₂ and iron-deficient conditions for 2 days. The fractionated proteins were separated by SDS-PAGE and electroblotted onto a PVDF filter, 20 μg of proteins being applied to each lane. The blotted filter was stained by Coomassie brilliant blue (lanes 1, 2 and 3) or incubated with the anti-HCR2 antiserum (lanes 4, 5 and 6). The bound antibody was detected as described in Materials and Methods. The arrow indicates the position of the HCR2 protein, and molecular weight (MW) markers are indicated on the left of the figure.

ferred to 20% CO₂, the length of the lag period for growth was about 1 day in each repeat of the experiments (Fig. 3B). A comparison of the polypeptides from cells grown under air and 20% CO₂ conditions suggested the presence of some specific polypeptides in those cells grown under 20% CO₂ for 2 days (data not shown). These two facts suggest that the expression of mRNA corresponding to high-CO₂ acclimatization appeared 1 to 2 days after transferring air-grown cells to 20% CO₂. Hence, a cDNA library was constructed for differential screening from those cells which had been grown under 20% CO₂ for 2 days.

Only two groups classified from the 78 clones were abundantly expressed under 20% CO₂ conditions in *C. littorale*. A search of the HCR1 amino acid sequence showed no significant homology to any other determined sequences, indicating HCR1 to be a novel group of genes responsible for extremely high-CO₂ and iron-deficient stress responses.

Some parts of the HCR2 amino acid sequence show significant similarity to gp91-phox, FRE1, FRE2 and Frpl (Fig. 2). The gp91-phox protein is a large subunit of human phagocyte oxidoreductase. This protein is a critical part of the membrane-bound oxidase of phagocytes, and is thought to be the terminal component of the respiratory chain that transfers a single electron from cytoplasmic NADPH across the plasma membrane to molecular oxygen on the exterior (Orkin 1989). The FRE1 gene has been cloned from the yeast, *Saccharomyces cerevisiae* (Dancis et al. 1992), and the Frpl gene has been identified in *Schizosaccharomyces pombe* (Roman et al. 1993). FRE2, which has significant amino acid homology with FRE1 (Georgatsou and Alexandraki 1994), also exhibits homology with HCR2. These authors assumed that the FRE1, FRE2 and Frp1 proteins were possible structural components of the respective cell-surface ferric reductase and may participate in the iron uptake by yeast. These proteins consist of several amino acid clusters, three of which are considered to be
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...ing involved in binding FAD and NADPH. The assignment of these clusters is based on the homology of gp91-phox with members of the ferredoxin-NADP⁺ reductase (FNR) family of oxidoreductases (Segal et al. 1992). Roman et al. (1993) have described that these three proteins may constitute a novel family of plasma membrane proteins responsible for the transport of electrons from cytoplasm to an extracellular substrate via FAD and heme intermediates. In *C. littorale*, no Cys-Gly dipeptide NADPH-binding segment was apparent in the HCR2 sequence. However, three other regions (containing FAD-binding and glycine-rich NADPH-binding domains) were conserved (Fig. 2). These facts indicate that HCR2 is a member of this flavocytochrome family.

Northern experiments show that HCR mRNAs were induced under 20% CO₂ conditions, but were not detectable under air conditions (Fig. 3). The mRNA levels decreased after transferring the cells from 20% CO₂ to air [Fig. 5 (II)], indicating that the expression of HCR1 and 2 was controlled by the CO₂ concentration. It was found that, under the 20% CO₂ conditions, an initial addition of excess FeSO₄ suppressed the induction of HCR mRNAs (Fig. 4), while addition during the induction period resulted in a decrease in the HCR mRNA levels [Fig. 5 (III)]. Although iron deficiency under air conditions slightly caused HCR1 expression (the mRNA was detected at day 6), no expression of HCR2 was observed at least for 10 days (data not shown). These results suggest that the expression of the HCR genes was regulated not only by CO₂, but also by the iron concentration in the medium. The HCR1 and 2 suppression by iron added under 20% CO₂ conditions was faster than that by transferring cells grown under 20% CO₂ and iron deficiency to air and iron-deficient conditions [Fig. 5 (II) and (III)], so that the expression of the HCR genes might have depended more directly on iron-deficient conditions than on a high-CO₂ concentration. One may argue that the acidification of the cytoplasm which might be caused by extremely high-CO₂ concentration would affect the HCR-mRNAs expression. However, Pronina et al. (in preparation) found that there was no difference in the cytoplasmic pH when *C. littorale* cells had been grown in air and air enriched with 20% CO₂. The sequence similarity between the HCR2 protein and the yeast ferric reductase proteins (Fre1, Fre2 and Frp1) suggests the possibility that the function of the HCR2 protein is associated with iron accumulation in *C. littorale* cells. After transferring to high-CO₂ and iron-deficient conditions, the accumulation of HCR1 was faster than that of HCR2 (Fig. 3, 4), indicating the involvement of different signal transduction pathways in their induction. On the contrary, the decrease in HCR1 mRNA was slower than that of HCR2 after transferring the cells to conditions of air or iron sufficiency (Fig. 5), suggesting the turnover rate or repression of the synthesis of HCR1-mRNA to be slower than that of HCR2.

It should be noted that, whereas the library was prepared from cells grown under 20% CO₂ in the medium containing a 10 μM FeSO₄ solution for 2 days, HCR2-mRNA expression was detected at day 3 after transfer to the same conditions (e.g. Fig. 3, 4). In the experiment shown in Fig. 3 and 4, an FeSO₄ solution was added to the medium just before starting cultivation. No special attention, however, was paid to the timing of FeSO₄ addition to prepare the algal materials used for screening the high-CO₂-specific cDNAs. In the culture medium used for such preparation, iron was added long before the start of the algal culture. As already mentioned, ferrous iron is readily oxidized and the iron content would decrease in the culture medium. Therefore, the earlier expression of HCR2 that was experienced during the screening of HCR clones might have been due to the earlier depletion of iron in the culture medium.

Yeast Fre1 protein has been immunodetected in the plasma membrane fraction (Lesuisse et al. 1996). The diaphorases in the marine diatom, *Thalassiosira weissflogii*, which may be involved in the reduction of Fe(III) to Fe(II), also exist in the plasma membrane (Jones and Morel 1988). The present results from immunoblotting show that HCR2 proteins are also associated with the membrane fraction. The time course for the accumulation of HCR2 polypeptide (Fig. 7A) is similar to that for the increase of its mRNA (Fig. 5) observed when cells were transferred from air to 20% CO₂ conditions. Both the levels of HCR2 mRNA (Fig. 5) and of HCR2 polypeptide (Fig. 7B) decreased when the cells were transferred to an iron-sufficient state. These results suggest that the transcriptional response to iron concentrations is quick and that HCR2 polypeptide is not stable and the turnover rate was rapid under the iron-sufficient conditions.

The expression of the ferric reductase genes, *Fre1*, and *Frp1*, in yeast was reportedly controlled by the concentration of iron in the growth medium (Dancis et al. 1990, Roman et al. 1993). This expression was induced by iron depletion and repressed by the addition of ferric iron, exhibiting regulation similar to that by the mRNA level. To investigate the relationship among HCR2 and yeasts Fre1, 2, and Frp1, we also measured the cell-surface ferric reductase activity in *C. littorale*. This activity was detectable, and the induction pattern of the activity was similar to that observed with the level of HCR2 mRNA (submitted). We thus infer that the gene product of HCR2 is ferric reductase, although no direct evidence is available.

Martin and Fitzwater (1988) have suggested that the iron concentration limits phytoplankton growth, and consequently reduces photosynthesis. The so-called iron hypothesis has been tested in some parts of the ocean (Martin et al. 1994, Coale et al. 1996). Iron is an essential element for the protein related to photosynthesis, respiratory electron transport, chlorophyll synthesis, and a number of other bio-
synthetic reactions (Geider and La Roche 1994). But most iron exists in the ferric form under aquatic and terrestrial conditions. Oxidation of ferrous iron results in a decrease in solubility at neutral pH and is not readily utilized by the organisms. The content of ferrous iron in the growth medium of *C. littorale* immediately decreased after the starting to bubble air containing 20% CO₂ and ferrous iron might be converted to ferric iron (data not shown). Thus, cells are generally being stressed by a deficiency in ferrous iron under these conditions. Pesheva et al. (1994) showed that the activity of photosynthesis increased on the first day after transfer to 20% CO₂. It is therefore possible that more iron is necessary under 20% CO₂ than air conditions for the synthesis of the iron containing proteins (e.g. cytochromes, ferredoxin and D1 protein).

The results presented in this paper suggest that both the CO₂ and iron concentration are involved in the induction of HCR genes, and that the HCR2 protein may be one of a family of plasma membrane ferric reductase. Further study is necessary to identify the function of the two HCR proteins in *C. littorale* and to clarify the reason why the expression of the HCR genes is induced by the different type of stress conditions such as iron limitation and extremely high-CO₂ concentrations.

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO). We are very grateful for the experimental assistance of Ms. Seiko Miura, and we thank Dr. Shigeki Harayama (Marine Biotechnology Institute) for his valuable advice and discussions.

References


Received September 10, 1997; Accepted November 14, 1997.