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The heme oxygenase gene (*pbsA*) in the red alga *Rhodella violacea* is discontinuous and transcriptionally activated during iron limitation

(rhodophyta/chloroplast/genome/intron/phycobilin)

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ABSTRACT **Heme oxygenase (HO) catalyzes the opening of the heme ring with the release of iron in both plants and animals. In cyanobacteria, red algae, and cryptophyceae, HO is a key enzyme in the synthesis of the chromophoric part of the photosynthetic antennae. In an attempt to study the regulation of this key metabolic step, we cloned and sequenced the *pbsA* gene encoding this enzyme from the red alga *Rhodella violacea*. The gene is located on the chloroplast genome, split into three distant exons, and is presumably expressed by a trans-splicing mechanism. The deduced polypeptide sequence is homologous to other reported HOs from organisms containing phycobilisomes (*Porphyra purpurea* and *Synechocystis* sp. strain PCC 6803) and, to a lesser extent, to vertebrate enzymes. The expression is transcriptionally activated under iron deprivation, a stress condition frequently encountered by algae, suggesting a second role for HO as an iron-mobilizing agent in photosynthetic organisms.**

In the complex pathway of tetrapyrrole biosynthesis, heme oxygenase (HO) catalyzes the opening of the heme (iron protoporphyrin IX) ring at the alpha methene bridge to form biliverdin IX, Fe^{3+}, and carbon monoxide. It is a highly conserved enzyme throughout the animal and plant kingdoms. In animals, HO is involved in a catabolic process: its primary role is to degrade hemoglobin in senescent red blood cells with the concomitant recycling of iron. HO from at least 12 animal tissues (reviewed in ref. 1) has been characterized. In higher plants and algae, HO is involved in a metabolic process, the synthesis of tetrapyrrole chromophores (2), phycobilins, and phytochromobilins. The phycobilins are the chromophores of the phycobiliproteins, light harvesting complexes of various photosynthetic organisms such as cyanobacteria and rhodophytes, where they are assembled in phycobilisomes (PBS), and cryptophytes. The phytochromobilins are the chromophores of the phytochromes, photoreceptors encountered in higher plants, chlorophytes, and presumably also in red algae. HO from one red alga, *Cyanidium caldarium* (3), has been extensively characterized. In higher plants, where the pathway of phytochrome synthesis (4) is proposed to be the same as for the related bilins in algae (2), no enzymatic characterization of this first metabolic step has been performed, although mutants deficient in phytochrome chromophore biosynthesis have been described in *Arabidopsis* (5, 6) and pea (7).

In mammals, HO is a microsomal enzyme; activity *in vitro* is associated with the electron donor NADPH-cytochrome P_{450} reductase (8, 9). Animal HO exists as two isoenzymes: HO-1 (32 kDa), inducible by a large number of environmental agents (including heme), and the constitutive HO-2 (34 kDa). In contrast to the animal HO anchored to the microsomal fraction, the algal enzyme is soluble and requires ferredoxin and ferredoxin-NADP reductase for activity (3). Algal heme oxygenase from *C. caldarium* has an apparent native molecular weight of 38 kDa (3) and is inducible by δ-aminolevulinic acid or another precursor of heme (10).

At the molecular level, several animal HO-1 and HO-2 genes or corresponding cDNA have been cloned and sequenced. The deduced amino acid sequences of HO-1 and HO-2 are about 40% similar. The 5′ untranslated region of HO-1 genes contains numerous regulatory sites presumably involved in its complex regulation (1). The HO gene has not yet been isolated from higher plants. In algae, an HO gene (called *pbsA* for phycobilin synthesis) has been identified by systematic mapping, cloning, and sequencing of the chloroplast genome of the rhodophyte *Porphyra purpurea* (11). The gene of *C. caldarium* has not been isolated, but the use of protein and RNA synthesis inhibitors has shown that the gene, in contrast to that of *P. purpurea pbsA*, is nuclear and that the protein is synthesized on cytoplasmic ribosomes (10). Two different HO genes, named *ho*, were identified in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by homology with *P. purpurea pbsA* (12). A bacterial gene product (HmuO), homologous to heme oxygenase has been reported in *Corynebacterium diphthriae* (13).

We have studied the regulation of phycobilisome synthesis in cyanobacteria and red algae in our laboratory (14–17). Although the apoprotein part of the pigment has received much attention in the marine red alga *Rhodella violacea,* nothing is known about the genes involved in the synthesis of the chromophores of the phycobilisomes. We report here the isolation and characterization of the *pbsA* gene from this organism. We demonstrate that this gene is chloroplastic, split into three distant exons, and encodes a polypeptide of 27 kDa homologous to other previously described heme oxygenases. Due to its position at a key step in a heme- and ferredoxin-dependent pathway, it was reasonable to presume that the gene for heme oxygenase could be regulated by iron availability. We therefore studied the effect of iron deprivation on the *pbsA* mRNA level and show a transcriptional activation in response to iron deprivation. This finding suggests an additional role for heme oxygenase as an iron-mobilizing agent in photosynthetic organisms during iron-stress conditions.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from either Eurogentech or Amersham. [α-^{32}P]dATP or dCTP (3,000

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: HO, heme oxygenase; cpDNA, chloroplast DNA; RT-PCR, reverse transcription–PCR.
Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF000717, AF000718, and AF000719).
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Ci/mmol; 1 Ci = 37 GBq), [α-^{33}P]dATP (1,000–3,000 Ci/mmol), Hybond N membrane, and multiprime kits were from Amersham. The kilobase sequencing system was from BRL. Plasmid pTZ18 was from Pharmacia. Enzymes were used according to the manufacturer's instructions.

Culture Conditions and Growth Media. The unicellular marine red alga *R. violacea* (strain 115-79) was obtained from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität (Göttingen Germany). Cells were grown photoautotrophically in sterile artificial seawater (18) with the addition of vitamin B_{12} at 25 μg/liter. Cultures were incubated at 21°C in glass culture flasks continuously flushed with sterile air, illuminated with fluorescent tubes (120 μM quanta/m^{-2}/s^{-1}) with a 16 hr light/8 hr dark photoperiod.

For iron regulation studies, growth was measure under the same conditions with different iron concentrations ranging from 10 μM (standard artificial seawater) to 0.05 μM $FeCl_3$.

DNA Purification, Genomic Library Construction, and Hybridization. Plastid DNA [chloroplast DNA (cpDNA)] from *R. violacea* was isolated by procedures described for chromophyte algae (19). Total or partial DNA libraries were constructed by ligation of *Hin*dIII- or *Eco*RI-digested plastid DNA into pTZ18R. *Escherichia coli* strain GT869 (20) was used as bacterial host. Standard methods (21) were used for subcloning, *in situ* colony hybridization and Southern blot analysis.

The heterologous probes were the total *pbsA* gene from *P. purpurea* (11) or a part of the gene encoding the last 175 amino acids, obtained by PCR. Homologous probes were either a 0.9-kb *Hin*dIII fragment from clone 1 (Fig. 1*A*) or a cDNA in the region covering the first exon (174 nt), the second exon (75-nt) and the first 42 nt of the third exon, obtained by reverse transcription–PCR (RT-PCR) (see below).

DNA Sequencing. The DNA sequences were determined by the dideoxyribonucleotide chain termination method (22) on single- or double-stranded DNA with the use of reverse or "-40" primers from M13 or with individual oligonucleotides (Genset, Paris) designed from the *R. violacea* sequence. All sequences were determined on both strands.

Walk-On Plastid DNA. To sequence the DNA region situated in the vicinity of the 6.8-kb *Eco*RI clone (clone 2 in Fig. 1*A*), we progressed along the plastid DNA by random cloning of *Pvu*II fragments (total digest of plastid DNA) in pTZ18R followed by amplification with a primer designed at the end of the *Eco*RI fragment and the reverse primer of pTZ18R. Using these two primers, we amplified a 0.8-kb fragment (PCR 1 in Fig. 1*A*); we then progressed by random cloning of *Hin*dIII fragments in pTZ18R followed by amplification with a primer designed at the end of the previous PCR 1 fragment and the reverse primer of pTZ18R. With these two primers we amplified a 1.2-kb fragment (PCR 2 in Fig. 1*A*).

Amplification of Large Fragments on Plastid DNA. To determine the respective location of each of the three exons of *R. violacea pbsA*, we attempted to amplify by means of the Expand Long Template PCR System from Boehringer Mannheim (supposed to amplify up to 30 or 40 kb DNA) the plastid DNA between exons, using 6 oligonucleotides shown in Figs. 1 and 2 and associated in the 12 relevant combinations: HO11 (5′-CTATGATATATGACACTAATTTAG-3′), HO12 (5′-CATTAAGCTTACTTCATCTTCAATA-3′), HO20 (5′-ATGAAAAGACATCAAGAAAGT-3′), HO19 (5′-TTCTAAACTAGATTTTCTATT-3′), HO15 (5′-CTTGTCTTGCCATTCTAATCC-3′), and HO18 (5′-GCTTTTA AGTTAAATATGAAA-3′).

RNA Isolation and Northern Hybridization. Total RNA from *R. violacea* was isolated as described for cyanobacteria (23). RNA (5–10 μg) was electrophoresed on 1.2% denaturing agarose gels in HF buffer (0.5 M Hepes/10 mM EDTA/16% formaldehyde) and transferred to nylon membrane with 20× standard saline citrate (SSC); blots were prehybridized, hybridized (42°C), and washed according to Damerval *et al.* (24).

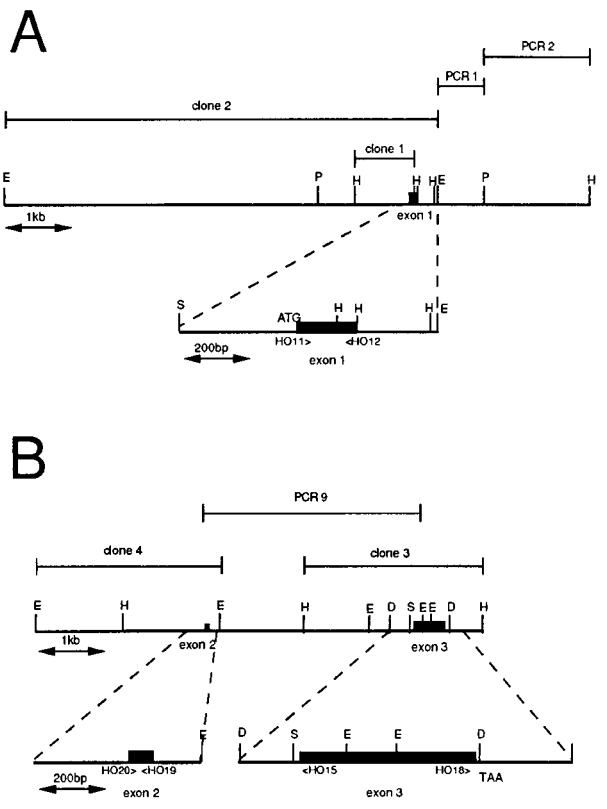

FIG. 1. Restriction maps of the two regions from *R. violacea* plastid DNA containing respectively exon 1 and exons 2 + 3 of the HO-encoding gene. Representative restriction sites: D, *Dra*I, E, *Eco*RI, H, *Hin*dIII, P, *Pvu*II, S, *Sau*3A. (*A*) The 8.8-kb region containing exon 1 obtained by cloning a 6.8-kb *Eco*RI fragment and its 2-kb flanking region by PCR walk on plastid DNA. (*Lower*) Sequenced region presented in Fig. 2*A*. (*B*) The 7-kb region containing exons 2 and 3. The whole was obtained by overlapping clones: a 2.7-kb *Hin*dIII clone (clone 3) containing exon 3, a 3.8-kb PCR product (PCR9) obtained between exon 2 and exon 3, and a 2.7-kb *Eco*RI clone (clone 4) isolated by screening a total *Eco*RI library with the 75-nt exon 2 obtained by PCR. (*Lower*) Sequenced regions presented in Fig. 2 *B* and *C*.

The probes used in Northern hybridizations were exon 1 (174 nt), exon 2 (75 nt) obtained by PCR amplification, and exon 3 (575 nt *Sau*3A–*Dra*I in Fig. 1*B*).

RT-PCR. To sequence the cDNA in the junction region between exons 1 and 3, we did coupled RT-PCR amplification. Total RNA (5 μg) from *R. violacea* was used with 0.02 pmol of oligonucleotide HO15 (Figs. 1*B* and 2*C*) as antisense primer, to synthesize the first strand with 400 units of reverse transcriptase (GIBCO) in the presence of 20 nanomol of each dNTP and 20 units of RNasin (Promega), an RNase inhibitor. After inhibition for 5 min at 95°C, the product of retrotranscription was amplified using HO11 (see Figs. 1*A* and 2*A*) as forward primer and HO15 as reverse primer, using Promega *Taq* polymerase in buffer and salt conditions recommended by the manufacturer. The program of the thermal cycler was the following: 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, and extension at 72°C for 1 min.

RT-PCR was also used to synthesize the cDNA corresponding to a putative pre-mRNA common to exon 2 and 3 separated by 3.8 kb. For cDNA synthesis HO15 was used as antisense primer, and PCR amplification was then performed with HO20 and HO15 as forward and reverse primers, respectively.

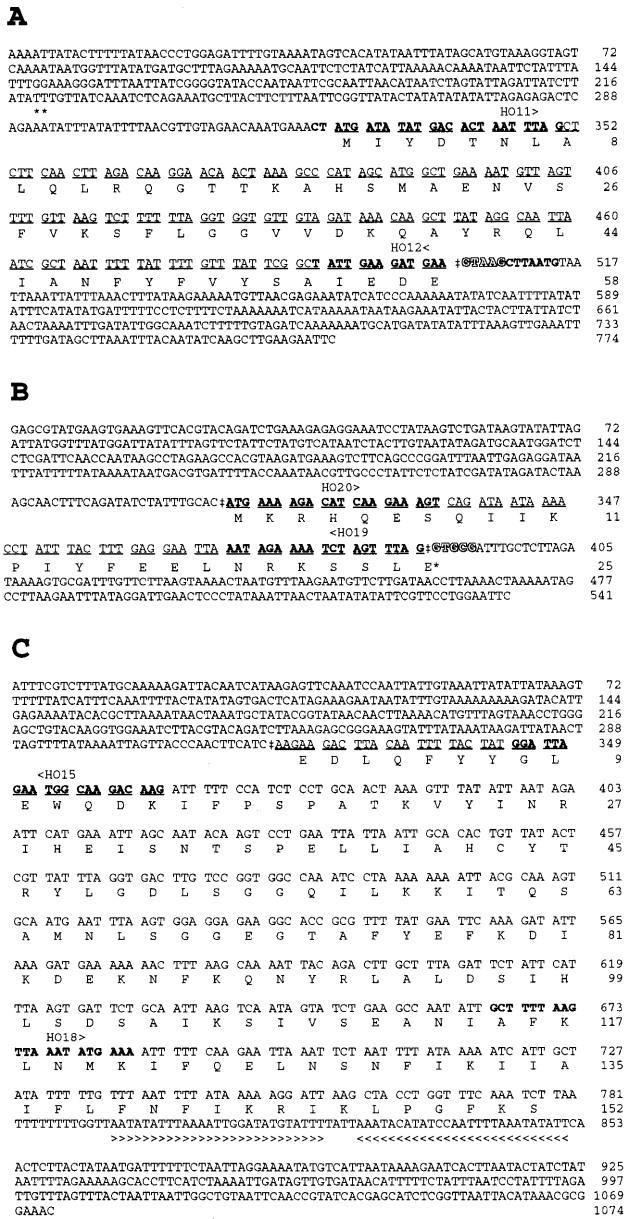

FIG. 2. DNA and amino acid sequences encoded by the exon 1 (*A*), exon 2 (*B*), and exon 3 (*C*) and flanking regions of the *pbsA* gene from *R. violacea*. DNA sequences underlined were confirmed by cDNA sequencing. The resulting RNA splice sites are marked by a †. Pentanucleotides characteristic of type II introns 5′ sequences are shaded. Nucleotides (or complementary sequences) in bold type correspond to oligonucleotides used as primers for PCR and RT-PCR. The 5′ end of mRNA is indicated by asterisks (*) in *A*. Glutamic E*25 in *B* is encoded by GAA after the splicing event joining exons 2 and 3. Inverted repeat at the 3′ end of *pbsA* is indicated by the arrowed line in *C*.

5′ mRNA Determination. Primer extension was used to map the 5′ terminus of mRNAs. The 25- or 21-mer oligonucleotides were used: HO12, HO19, and HO15, respectively complementary to exon 1, exon 2, and exon 3 (see Fig. 2). A modification of the method described (21) was followed: 30 μg of total RNA from an exponential culture of *R. violacea* was annealed overnight at 30°C (after 10 min denaturation at 85°C) with 10 pmol of each primer in 40 mM Pipes (pH 6.4), 1 mM EDTA (pH 8), 0.4 M NaCl, and 80% deionized formamide. After ethanol precipitation, the hybrid was incubated in 10 μl with 400 units of superscript reverse transcriptase (GIBCO) in the appropriate buffer in the presence of 1 mM DTT, 0.15 μM dCTP, dGTP, and DTTP, and 10 μCi of [^{33}P]dATP. After 10 min of labeling, extension was performed fo r 2 h by addition of 10 μM of each dNTP. Nonhybridized RNA was digested for 30 min by 0.5 μg of RNase. The samples were then ethanol-precipitated and loaded on a sequencing gel.

RESULTS

Gene Cloning and Determination of Nucleic Acid Sequences. The expected phylogenetic relationships between the two rhodophytes *R. violacea* and *P. purpurea* led us to attempt isolation of the *pbsA* gene of *R. violacea* by means of heterologous DNA hybridization experiments. A probe containing the *pbsA* gene of *P. purpurea* (11) was hybridized to Southern blots of nuclear and plastid (cpDNA) DNA fractions of *R. violacea* digested with different restriction enzymes (data not shown). Only the cpDNA showed positive signals, and the most appropriate enzyme was *Hin*dIII, giving two positive fragments of 2.7 and 0.9 kb. The 0.9-kb fragment was first isolated from a total *Hin*dIII library constructed in pTZ18R (clone 1 in Fig. 1*A*). This *Hin*dIII fragment was then used as an homologous probe to screen a total *Eco*RI library in pTZ18R where a 6.8-kb insert clone (clone 2 in Fig. 1*A*) was selected, in agreement with the result obtained with the heterologous *P. purpurea* probe on Southern blotting.

The DNA sequence of 774 nt of this region (Figs. 1*A* and 2*A*) revealed the presence of an ORF that corresponded to only the first 58 amino acids of the *R. violacea pbsA* gene, as confirmed by similarity with the protein deduced from the *P. purpurea* gene (GenBank accession no. P51271). This sequence provided an indication of the presence of an intron within the heme oxygenase gene of *R. violacea*. Analysis of the region downstream from the *Eco*RI site (Fig. 1*A*) was performed by PCR walking on the cpDNA, and 2 kb were thus cloned (PCR1+ PCR2, Fig. 1*A*). The remaining part of the *pbsA* gene was not encoded within this 2-kb region, as shown by hybridization with the *P. purpurea* probe, and by partial sequencing.

A 2.7-kb *Hin*dIII fragment (clone 3, Fig. 1*B*) was further isolated from a partial pTZ18R library by heterologous hybridization with a probe containing the 3′ part of the *P. purpurea pbsA* gene. From this second region (Fig. 1*B Right*) a continuous DNA sequence of 1,074 bp was obtained (see Fig. 2*C*). By comparison with *P. purpurea* HO, we identified in this sequence the coding information for the C-terminal 152 residues. Between these two coding regions (Fig. 2 *A* and *C*), a gap of 25 amino acids was observed in the *R. violacea* polypeptide in comparison to other HOs. To verify the polypeptide sequence of *R. violacea* HO deduced from the sequencing of the two plastid DNAs already cloned, we synthesized and sequenced the cDNA corresponding to the junction of these regions (see *Materials and Methods*). A product of 300 bp was obtained and sequenced. The sequences of the entire exon 1 and the beginning of the distal exon were recovered, but separated by a new coding sequence of 75 nt that covered the gap of the 25 "missing" amino acids, thus confirming the presence of a third central exon.

Using oligonucleotides HO20 and HO19 (Figs. 1*B* and 2*B*) we synthesized the central 75-nt exon 2 and used it as a probe against a Southern blot of cpDNA. We thus detected a positive 2.7-kb *Eco*RI fragment and screened a total *Eco*RI library into which a clone (clone 4, Fig. 1*B*) containing this insert was found and partly sequenced. The sequence of exon 2 and flanking regions is presented in Fig. 2*B*.

Positioning the Three Different Exons Within the *R. violacea* Chloroplast Genome. To determine the distance between the three exons of *R. violacea pbsA*, we attempted to amplify the plastid DNA present between the different coding regions using the 12 relevant combinations of oligonucleotides in the two orientations of the three exons by means of an Expand Long Template PCR System (Boehringer Mannheim). Only

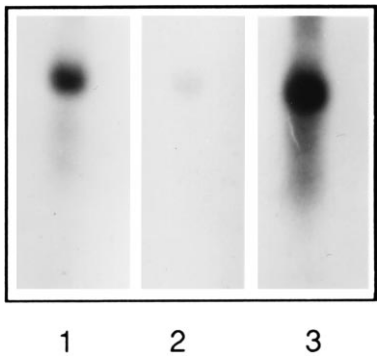

FIG. 3. Autoradiogram of Northern blots of total RNA (5 μg) from *R. violacea* hybridized with *pbsA* exon 1 (lane 1), exon 2 (lane 2), and exon 3 (lane 3) probes. Autoradiography was for 20 h for lanes 1 and 3, and 72 h for lane 2.

the pair of oligonucleotides HO15 and HO20 resulted in an amplification product of 3.8 kb (see PCR 9 in Fig. 1*B*) that was cloned and sequenced in the vicinity of exon 2. This result confirmed the distance of about 3.8 kb and orientation between exon 2 and exon 3. The fact that no amplification product was obtained when HO11 and HO12 were used allows us to suggest that the distance between exon1 and exons 2–3 is more than 20 kb.

Transcriptional Analysis. To identify the transcription product of the HO gene parts, we used specific DNA probes of exons 1, 2, and 3 of the *pbsA* gene in Northern blot analysis. The three probes hybridized with a common 0.85-kb mRNA transcript (Fig. 3) confirming the assumption of a functional gene represented by these three exons.

We attempted to characterize other RNA representing pre-mRNA species by hybridization with the three probes specific to exons 1, 2, and 3. Growing cells in the cold, conditions which should slow down splicing (25), and long exposure of the autoradiogram did not permit the detection of any other significant transcript. We also attempted to detect a common pre-mRNA between exons 2 and 3 by RT-PCR using oligonucleotides HO20 and HO15 (Fig. 1*B*); no such species was obtained.

5′ End of mRNA. The 5′ end of the *pbsA* transcript was mapped by primer extension with oligonucleotides hybridizing with exon 1 (HO12), exon 2 (HO19), and exon 3 (HO15). The three oligonucleotides give a 5′ end for the mature mRNA occurring 37 or 38 bp before the initiating ATG (nucleotides 292 and 293; see the ∗ in Fig. 2*A*).

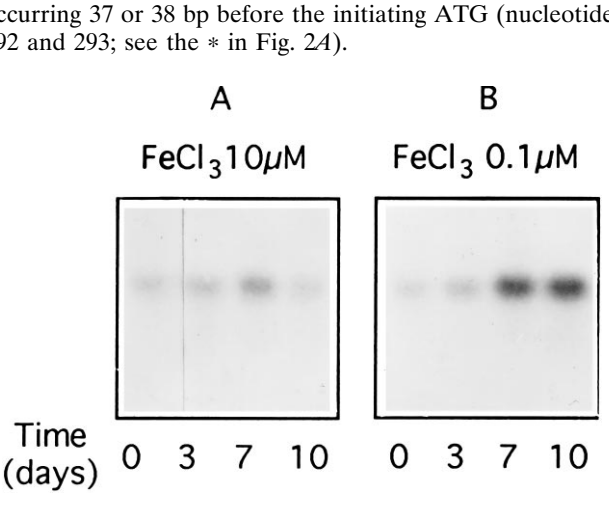

FIG. 4. Autoradiogram of Northern blots of total RNA (5 μg) from *R. violacea* grown either in 10 μM (*A*) or in 0.1 μM $FeCl_3$ (*B*) in artificial sea water medium. Hybridization was performed with *pbsA* exon 1 probe.

***pbsA*, a Gene Activated by Iron Stress.** To determine the effect of iron on *pbsA* transcription, cells were grown for several generations in artificial sea water medium containing iron concentrations ranging from 10 to 0.05 μM $FeCl_3$. Iron limitation did not affect growth rate; the doubling time was ≈26–30 h for both control and deficient cells. However, for iron concentrations below 0.2 μM, cell size and pigment content (chlorophyll *a* and phycobiliproteins) decreased. *R. violacea* cells responded to a reduction of Fe concentration from 10 μM (Fig. 4*A*) to 0.1 μM (Fig. 4*B*) by increasing the levels of the *pbsA* transcript. We assume that the level of HO activity is consequently increased.

DISCUSSION

In this paper we present results concerning the isolation, cloning and sequencing of the HO gene, *pbsA,* from the unicellular red alga *R. violacea*.

The coding region of the *R. violacea pbsA* gene is highly similar to the corresponding genes from a variety of organisms (see Fig. 5). The *R. violacea* HO deduced from the nucleotide sequence shows identity of 59%, 52% ,and 43% with *P. purpurea*, *Synechocystis* sp. strain PCC 6803 (product of gene 1, sll1184), and *Synechocystis* sp. strain PCC 6803 (product of gene 2, sll1875), respectively; taking into account conservative substitutions of amino acids, similarities reach 76%, 73%, and 65% respectively. The HO from these four organisms are very close in size (27–29 kDa), the shortest being the *R. violacea* enzyme, and significantly shorter than the heme-binding component (38 kDa) of *C. caldarium* estimated by gel filtration on a Sephadex G75 column (3). Similarity is greatest around the

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          10        20        30        40        50        60        70
1                           MIYDTNLALQLRQGTTK-AHSMAENVSFVKSFLGGVVDKQAYRQLI
2                           MV--SSLANELREGTTS--HSMAENVSFVKSFLGGVVDKKSYRKLI
3                            M-SVNLASQLREGT-KKSHSMAENVGFVKCFLKGVVEKNSYRKLV
4                           M---TNLAQKLRYGTQQ-SHTLAENTAYMKCFLKGIVEREPFRQLL
5                     METSQPHNAESMSQDLSELLKEAT-KEVHEQAENTPFMKNFQKGQVSLHEFK-LV
6   MSAEVETSEGVDESEKKNSGALEKENQMRMADLSELLKEGT-KEAHDRAENTQFVKDFLKGNIKKELFK-LA
                                        **  ** **    *  ***  *** ** * *    ** *

          80        90       100       110       120       130       140
1   -ANFYFVYSAIEDE†MKRHQESQIIKPIYFE-ELNRKSSLE†EDLQFYYGLEWQDKIFPSPATKVYINRIHEIS
2   -ANLYFVYSAIEEE ILLNKDHPAIKPIYFT-ELNRKTSLA KDLNYYYGPDWINIIEPSSATQVYVNRIHNIG
3   --NLYFVYSAMEEE MAKFKDHPILSHIYFP-ELNRKQSLE QDLQFYYGSNWRQEVKISAAGQAYVDRVRQVI
4   -ANLYYLYSALEAA LRQHRDNEIISAIYFP-ELNVTDKLA EDLTYYYGPNWQQIIQPTPCAKIYVDRLKTIA
5   TASLYFIYSALEEE IERNKDNPVYAPVYFPMELHRKAALE KDLEYFYGSNWRAEIPCPEATQKYVERLHVVG
6   TTALYFTYSALEEE MDRNKDHPAFAPLYFPMELHRKEALT KDMEYFFGENWEEQVQCPKAAQKYVERIHYIG
        ** ****    *    *      ***  **     *   ** **** **   *     *  ** **  *

          150       160       170       180       190       200       210
1   NTSPELLIAHCYTRYLGDLSGGQILKKITQSAMNL-SGGEGTAFYEFKDIKDEKN---FKQNYRLALDSIHL
2   NKQPELLVAHAYTRYLGDLSGGQVLKKIARGAMNL-SDERGTKFYDFDEIEDDKI---FKNNYRSALDTIPL
3   ATAPELLVAHSYTRYLGDLSGGQILKKIAQNAMNLH---DGTAFYEFADIDDEKA---FKNTYRQAMNDLPI
4   ASEPELLIAHCYTRYLGDLSGGQSLKNIIRSALQLP-EGEGTAMYEFDSLPTPGDRRQFKEIYRDVLNSLPL
5   KKHPELLVAHAYTRYLGDLSGGQVLKKIAQKALQLPSTGEGLAFFTFDGVSNATK---FKQLYRSRMNALEM
6   QNEPELLVAHAYTRYMGDLSGGQVLKKVAQRALKLPSTGEGTQFYLFENVDNAQQ---FKQLYRARMNALDL
      ******* ************ ****   ** *    **   * *  *         *** **  ** * *

          220       230       240       250       260       270       280
1   SDSAIKSIVSEANIAFKLNMKIFQEL--NSNFIKIIAIFLFNFIKRIKLPGFKS----------------
2   SDEQVQNVVAEANISFTLNMKMFEEL--NSSIVKIITMIIVSTVRKFTTLKSILATAD------------
3   DQATAERIVDEANDAFAMNMKMFNELEGNLIKAIGIMVFNSLTRRRSQGSTEVGLATSEG----------
4   DEATINRIVEEANYAFSLNREVMHDLEDLIKAAIGEHTFDLLTRQDRPGSTEAR-STA-GHPITLMVGE-
5   DHATKKRVLEEAKKAFLLNIQVFEAL-QKLVSKSQENGHAVQPKAELRT--RSVNKSHENSPAA---GGE
6   NMKTKERIVE-ANKAFEYNMQIFNELDQAGSTLARETLEDGFPVHDGKGDMRKCPFYAAEQ-DKG-LEGS
          **  *  **  *  *   *

          290       300       310       320
1   ------------------------------------          235 AA
2   ------------------------------------          236 AA
3   ------------------------------------          240 AA
4   ------------------------------------          250 AA
5   RTSRMQADMLTTSPLVRWLLALGFIATTVAVGLFAM          294 AA
6   LSLP-TSYAVLRKPSLQFILAAGVALAAGLLAWYYM          314 AA
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FIG. 5. Comparison of amino acid sequences derived from the homologous heme oxygenase genes from different sources: line 1, *R. violacea*, this work; line 2, *Porphyra purpurea* (26); line 3, *Synechocystis* sp. strain PCC 6803, gene sll1184 (12); line 4, *Synechocystis* sp. strain PCC 6803, gene sll1875 (12); line 5, chicken HO-1 (27); and line 6, human kidney HO-2 (28). Stars indicate identical or conservative amino acid residues in the six different sequences. The † in *R. violacea* HO indicate the break points of exons 1–2 and exons 2–3.

active site that contains highly conserved hydrophobic residues 148–173 (Fig. 5) and includes the essential His-154 supposed to bind heme (9). Animal HO are longer than algal polypeptides; they contain a C-terminal tail that is involved in the anchoring of the protein to the microsomal fraction, a tryptic digest of the C terminus resulting in a catalytically active soluble enzyme. Furthermore, HO-2 has a N-terminal extension of 20 or so residues compared with the HO-1. In the region where HO from *R. violacea* and animals are aligned, a significant (around 33%) similarity is observed. Similarity in the same range is found between *R. violacea pbsA* and *C. diphteriae hmuO* (13) gene products.

Interestingly, we have demonstrated that in *R. violacea*, *pbsA* is split into three exons whereas in *P. purpurea* (11), the other rhodophyte from which the gene has been isolated, *pbsA* is continuous. Furthermore, *R. violacea pbsA* is spread over the chloroplast genome, exon 2 and exon 3 being separated by 3.8 kb as determined by PCR. The location of the first exon with respect to the other two still remains unknown, though PCR experiments indicate that it is at least 20 kb away.

These results suggest that a complex mechanism produces the mature transcript. We could propose a trans-splicing mechanism between the first exon and exons 2 and 3, taking into account the possible distance (presumably >20 kb) between them. Such a splicing mechanism has already been described in chloroplast genes such as *psaA1* of *Chlamydomonas reinhardtii* (29) or *rpsl12* of tobacco (30) and in plant mitochondrial genes (reviewed in ref. 31). On the contrary, for exons 2 and 3, separated by 3.8 kb and in the same orientation, joining of the two exons could be possible from a single pre-mRNA subject to cis-splicing, though a common transcript of ≈4 kb was not detected by either Northern hybridization or RT-PCR. The probable occurrence of such a complex splicing mechanism (involving cis- and trans-splicing) has been previously reported (reviewed in ref. 32).

The *pbsA* gene of *R. violacea* is, to our knowledge, only the second example together with the *R. violacea rpeB* gene (14) of a split gene in a rhodophyte plastid genome. The detection of chloroplast introns only in *R. violacea* and not in the other rhodophytes studied so far suggests that either (according to the "introns late" theory), they entered the *R. violacea* plastid after the separation from other rhodophytes or that (according to the "intron early" theory) they were lost in the other red algae studied previously (see ref. 33 for review).

Flanking regions of exons 1, 2 and 3 on the cpDNA (see Fig. 2 *A*, *B*, and *C*) reveal some features of group II introns (33): exon 1 is followed by GTAAG and exon 2 is followed by GTGCG, similar to the 5-nt GTGYG encountered at the 5′ end of group II introns. At the 3′ end of the introns a C is found immediately preceding exon 2 and exon 3, and the A is found 8 nt upstream from exon 2 and exon 3, is presumably involved in the lariat formation, also specific to group II introns (34).

The 5′ end of mature mRNA determined by primer extension with three different primers is situated at nucleotides A 292 and 293 (Fig. 2*A*). Sequences weakly similar to −35 (nt 251–256) and −10 (nt 273–278) promoter elements of bacterial and plastid genes are found upstream from these 5′ end nucleotides. However, we cannot assert that this is the transcription initiation site rather than a processing or splicing site. Downstream from exon 3 (Fig. 2*C*), an inverted repeat of 27 nt able to form a stable stem configuration could be a barrier against exonucleolytic attack of the mRNA and/or correspond to a transcription terminator. The length of ≈850 nt of the mature RNA obtained by Northern hybridization is in agreement with the size determined between the 5′ end of the mature mRNA and this putative 3′ end.

We have shown that the *pbsA* gene (shown at the mRNA level and presumed at the enzyme activity level) from *R. violacea* is more highly expressed under iron limitation. Iron is of special importance for photosynthetic organisms as it is present as a cofactor in many protein complexes of the photosynthetic apparatus such as reaction centers and cytochromes. Iron is also necessary for the synthesis (via heme) of bilin pigments. The biological availability of iron is often limiting due to the low solubility of Fe^{3+} above neutral pH in marine and fresh waters (35). Algae, as cyanobacteria, must increase their iron content to compensate for growth and cell division. As a necessity, they have evolved a number of responses to deal with frequently occurring iron deficiencies. HO transcriptional activation could represent one of these responses. This physiological response, never reported before in photosynthetic organisms, suggests a new and interesting role for the product of *pbsA* as a mobilizing agent during iron limitation conditions. We suggest that the *pbsA* gene product, besides its anabolic function in bilin synthesis, plays a role in a catabolic process by removal of iron from heme.

The transcriptional regulation of *pbsA* by iron limitation remains to be studied at the molecular level. The current model involving a Fur-repressor binding to "Fur boxes" for turning off the expression of iron regulated genes in microorganisms (reviewed in ref. 36) may be considered, although no such sequence is present in the vicinity of the *pbsA* putative promoter region. Alternatively, the mechanism involved can be totally different for *pbsA* where a basal level of expression is needed in iron-replete conditions in contrast to Fur-regulated genes in bacteria, whose transcription is either on (low iron) or off (iron excess).

The unambiguous chloroplastic origin of *P. purpurea* and *R. violacea pbsA* genes has to be compared with the very likely nuclear location of the corresponding gene in another red alga, *C. caldarium* (10). This result might give useful information about evolution: in *R. violacea* and *P. purpurea* the export of genes from the chloroplast to the nucleus is less extensive than in Cyanidiophyceae. In addition, in higher plants and presumably also in the green alga *C. reinhardtii*, the HO encoding gene has not been found on plastid DNA so far sequenced and is therefore probably also nuclear.

Thus the role of HO appears to be multiple: in pathogenic bacteria, its enzymatic activity is essential in the processes of host infection where iron is needed (13); in animals, HO is not only used for degrading hemoglobin but also serves in modulation of oxidative stress and in the regulation of cell growth and differentiation (1); in plants and other photosynthetic organisms such as algae and cyanobacteria, it could have a dual role: (*i*) biosynthesis of bilin chromophores for photosynthetic antennae or photoreceptor synthesis and (*ii*) release of iron from available cell components under deprivation conditions.

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