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The Cloning of Two Hydrogenase Genes from the Green Alga *Chlamydomonas reinhardtii*

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Introduction

The green alga *Chlamydomonas reinhardtii* is capable of photoproducing H_2 gas from water (Greenbaum et al. 1983), exogenous substrates (Gibbs et al. 1986) or endogenous substrates (Gfeller and Gibbs 1985). Hydrogen production is catalyzed by hydrogenases, enzymes that are extremely sensitive to O_2 (Ghirardi et al. 1997). Practical applications will require engineering a hydrogenase that can operate in the presence of oxygen. Our laboratory has been attempting to clone the *C. reinhardtii* reversible hydrogenase. Once cloned, it can be subjected to a variety of mutagenesis methods to generate O_2-tolerant mutants. Indeed, mutant bacterial organisms containing hydrogenases that are able to function at higher O_2 concentrations have been described (McTavish et al. 1995), suggesting that such enzymes are amenable to manipulations that affect O_2 tolerance.

Iron-only hydrogenases, many of which catalyze H_2 production *in vivo*, contain only FeS clusters at their activity sites. These enzymes, found in anaerobic bacteria and green algae, contain three or two 4Fe4S centers and one 2Fe2S center (Adams 1990). The catalytic site of the enzyme consists of a 4Fe4S and a 2Fe2S and is called the H-cluster. Iron-only hydrogenases usually have high specific activity but are easily inactivated by O_2 and CO. The *C. reinhardtii* hydrogenase is an Fe-only enzyme and has been isolated to purity by Happe and Naber (1993), who reported a 24-amino acid residue, N-terminal sequence of the enzyme. In this paper, we describe an approach to clone the *C. reinhardtii* hydrogenase based on the high degree of homology between previously-cloned Fe-only hydrogenases (mostly bacterial). Regions of homology found in these enzymes were used to guide the design of gene-specific 5' and 3'-end primers that were then used to amplify the catalytic region of the hydrogenase gene from a pool of algal mRNA. The amplified product was then used as a probe to isolate the complete gene from a *C. reinhardtii* cDNA library.

Materials and Methods

Cell growth, anaerobic induction, mRNA extraction, reverse transcription and RT-PCR

Chlamydomonas reinhardtii strain cc125, containing the cw15 cell wall-less mutation, was grown in the light in TAP (Harris, 1989) to a concentration of about 15µg Chl/ml. Harvested cells were anaerobically induced as previously described (Ghirardi et al. 1997) in phosphate

buffer and immediately lysed using binding buffer (S.N.A.P kit, Invitrogen Inc.). The pooled total RNA was further enriched for poly-A^+ RNA (FastTrack 2.0 kit, Invitrogen Inc.). The resulting mRNA was reverse-transcribed at 50°C using poly-dT_{15} primers and random hexamers (Superscript II kit, Life Technologies Inc). An aliquot of the cDNA was PCR-amplified in 30 mM Tricine, pH 8.4 (20 °C), 3.5 mM $MgCl_2$, 5 mM ß-mercapto-ethanol, 0.01% gelatin, 0.01% Igepal CA-630, 1.2 mM dNTPs, and 1.5 µM primers with annealing at 58 °C for 30 cycles.

cDNA library screening and lambda phage plasmid excision

The PCR-amplified products were cloned onto the pUC19 vector. An amplification product isolated from clone RC53_43 was fluorescently labeled using digoxigenin-11-dUTP (Roche Molecular Biology) and used to screen a lambda ZAP II *C. reinhardtii* cDNA library (kindly provided by John Davies, now at Exelixis Inc.). The cDNA inserts were retrieved from positive plaques as pBluescript SK(-) plasmids (Rapid Excision kit, Stratagene Inc, with modifications). The cDNA clone containing the amplified product was designated Hyd A.

Expressed sequence tag (EST) amplification and cDNA library screening

Two primers, BE5P1 and BE3P1, were designed for amplification of a *C. reinhardtii* EST, shown to have a high degree of homology to other Fe-only hydrogenases in the GenBank database. The primers were then used to amplify the EST from a sample of the *C. reinhardtii* cDNA library (Failsafe PCR kit, Epicentre Inc.). Clone EST_26, containing the amplified nucleotide sequence of the EST, was then used to generate digoxigenin-labeled probes for cDNA library screening. A strongly-hybridizing clone, designated Hyd B, was retrieved in pBluescript SK (-).

Northern blot analysis

Five µg of total RNA were fractionated by electrophoresis on 1.2% formaldehyde agarose gels, blotted onto Nytran N^+ membranes, and fixed by UV crosslinking and baking for 2h at 80 °C. Membranes were prehybridized in 6 x SSC (900 mM NaCl, 90 mM Na citrate) buffer, 10% dextran sulfate, 50% formamide, 1 x Denhardt's solution, 0.1 % SDS and 100 µg/mL denatured salmon sperm DNA at 42 °C for ≥ 1 h. Denatured ^{32}P labelled probe was added and the blots were washed at medium stringency (0.5 x SSC, 0.5 % SDS at 58°C) after overnight hybridization. Membranes were exposed to storage phosphor screens (Kodak Inc.) and scanned with a STORM 860 PhosphorImager (Molecular Dynamics Inc.). Quantitation was performed with the ImageQuant software provided. The blots were subsequently stripped and reprobed for ribosomal RNA to normalize the HydA and HydB transcript levels for loading.

H_2-Evolution Assay

MOPS buffer (50 mM, pH 6.8) was added into a water-jacketed chamber (a 2.5 ml volume held at 25° C) and equipped with two Clark electrodes (YSI 5331, Yellow Springs, OH), one poised for the measurement of H_2 and the other for O_2 production (Ghirardi, et al., 1997). The O_2 concentration in the cuvette was set close to zero with Ar, and 200 µl of anaerobically induced cell suspension were injected into the buffer. The cell suspension was illuminated (320 µE m^{-2} s^{-1}, PAR incandescent light filtered through 1% $CuSO_4$) for three minutes. The initial rates of H_2 production were estimated from the initial slope of the curves.

Results and Discussion

RT-PCR amplification of the hydrogenase active site

A multiple sequence alignment of Fe-only hydrogenases from *Clostridium pasteurianum*, *Trichomonas vaginalis,* and *Desulfovibrio vulgaris* was obtained and used as input to the CODEHOP algorithm (http://www.blocks.fhcrc.org/codehop. html) to generate potential primers for the PCR amplification reaction. The chosen primers, RC5 and RC3, had 64 and 32 degeneracies, respectively. An anaerobically induced sample of *C. reinhardtii* provided the source mRNA material for direct RT-PCR amplification. A double band centered around 800 basepairs was evident in the lanes originating from induced mRNA but not in the controls (not shown). The double band was excised and blunt-end cloned into the vector pUC19. One of the resulting clones, RC53_43, showed all four strictly conserved cysteines that are involved in binding the catalytic 4Fe-4S cluster to a 2Fe-2S cluster (the H-cluster). It contained a 45-amino acid insertion between H-cluster motifs 2 and 3, similar to a 28-amino acid insertion at the same location in putative Fe-only hydrogenase clones from *Scenedesmus obliquus* (Florin et al. 2001; Wünschiers et al. 2001). Figure 1 shows that the expression of this active site sequence is dependent upon anaerobiosis, supporting its possible role as a hydrogenase.

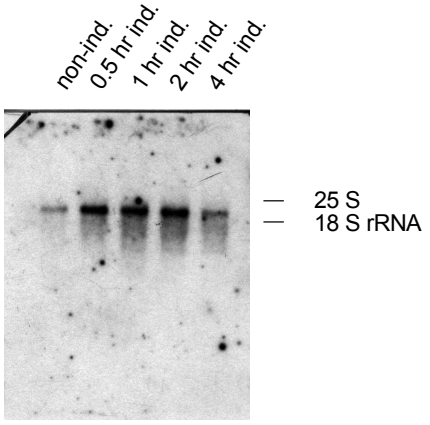

Figure 1. Northern blot of mRNA isolated from non-induced and anaerobically induced *C. reinhardtii* cells, and probed with RC53_43.

Retrieval of full-length Hyd A and Hyd B clones

The active site clone RC53_43 was used as a probe to retrieve a full-length clone from the cDNA library. From 30,000 lambda plaques plated, 40 hybridized to the probe. The lambda plaques were subject to an *in vivo* excision procedure to obtain the cDNAs as pBluescript SK (-) plasmids. Restriction site analysis and DNA sequencing proved one of the clones, Hyd A, to be 100% identical to an unpublished clone in GenBank, denoted hyd1 (Accession no. AF 289201). Hyd A was retrieved as a 2.45-kilobase cDNA exhibiting an open reading frame (ORF) encoding 497 amino acids. It also contained the previously reported 24-amino acid N-terminus (Happe & Naber 1993), preceded by a 56 residue leader peptide that directs the protein to the chloroplast stroma.

The deduced amino acid sequence of clone RC53_43 revealed a close match in a BLAST search to an expressed sequence tag (EST) from *C. reinhardtii*. We designed primers to amplify this EST using the cDNA library. The resulting clone EST_26 was identical in

nucleotide sequence to the original EST. However, it was distinctively different from the active site clone RC53_43 in that it contained a unique restriction site not found in the latter. Clone EST_26 was then used as a probe to again screen the cDNA library, which led to the retrieval of a full-length clone, Hyd B. The Hyd B cDNA is slightly larger in size than Hyd A and is predicted to encode a 505 residue enzyme. An online service (http://www.cbs.dtu.dk/services/ChloroP-1.0.html) predicted the presence of a chloroplast sequence with a cleavage site 16 amino acids after the start of the ORF, which may be too short for a signal peptide.

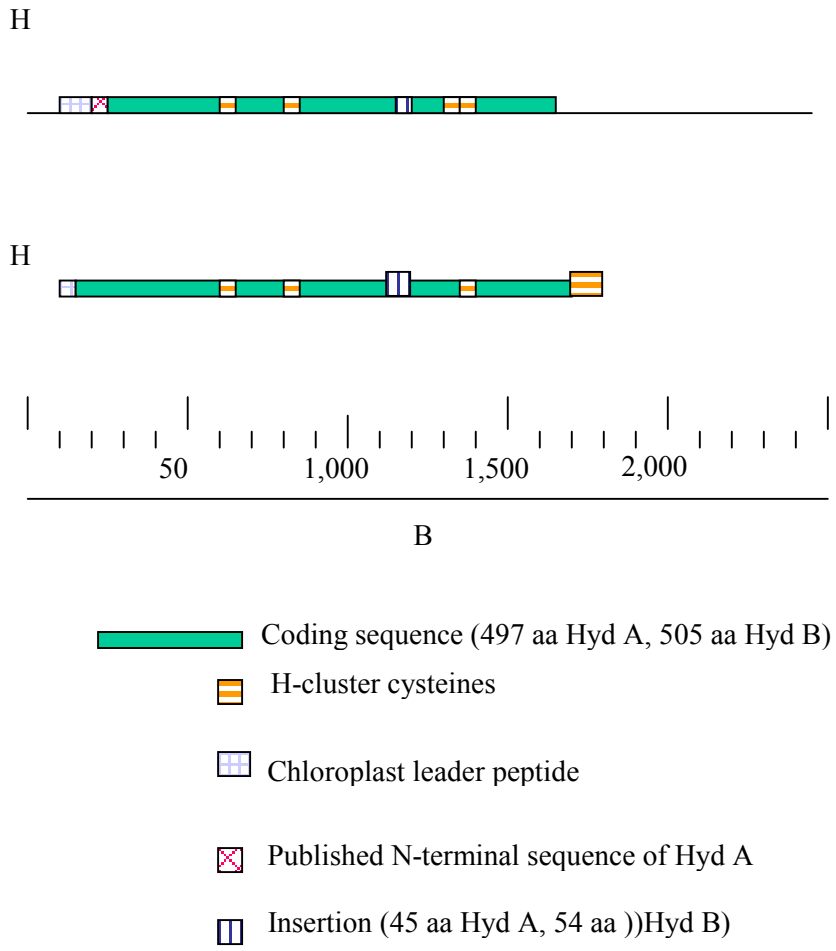

Figure 2. Comparison of the *Chlamydomonas reinhardtii* Hyd A and Hyd B transcripts encoding two different Fe-only reversible hydrogenases

Figure 2 shows a comparison between the Hyd A and Hyd B transcripts. A comparison of the newly isolated clones to Hydrogenase I from *Clostridium pasteurianum* (Meyer & Gagnon 1991) and two hydrogenase clones from *Scenedesmus obliquus* (Florin et al. 2001; Wünschiers et al. 2001), as expected, places them in closer relationship with the latter ones (Accession nos. AJ271546 and AF276706). *S. obliquus* and *C. reinhardtii* Fe-only hydrogenases share two characteristic features found only in the green algal enzymes: an amino acid residue insertion (16 residues in *S. obliquus*; 45 residues in *C. reinhardtii* Hyd A, and 54 residues in *C. reinhardtii* Hyd B) between H-cluster motifs 2 and 3, and the lack of

the more distal 4Fe-4S clusters normally found in all bacterial and protozoan Fe-only hydrogenases (Cammack 1999). The biological implications of these unique features are not known, at present.

Analysis of Hyd A and Hyd B mRNA levels upon anaerobic induction

The expression of the two *C. reinhardtii* hydrogenases was studied by Northern blot analyses and compared to the amount of enzyme activity after incubation under anaerobic conditions. Figure 3A shows that a small amount of Hyd A mRNA is detected under aerobic conditions (0 h) and that it increases about 4-fold during a 4-h anaerobic incubation period. These data are in agreement with measurements of light-dependent hydrogenase activity (Fig. 3B). The levels of Hyd B mRNA, on the other hand, seem to change little during the anaerobic induction treatment and do not correlate with H_2-production activity. The results suggest that Hyd A encodes the chloroplast reversible Fe-only hydrogenase linked to photosynthetic electron transport through ferredoxin. The physiological role of the hydrogenase encoded by Hyd B is not known at present, but it may be a second chloroplast protein.

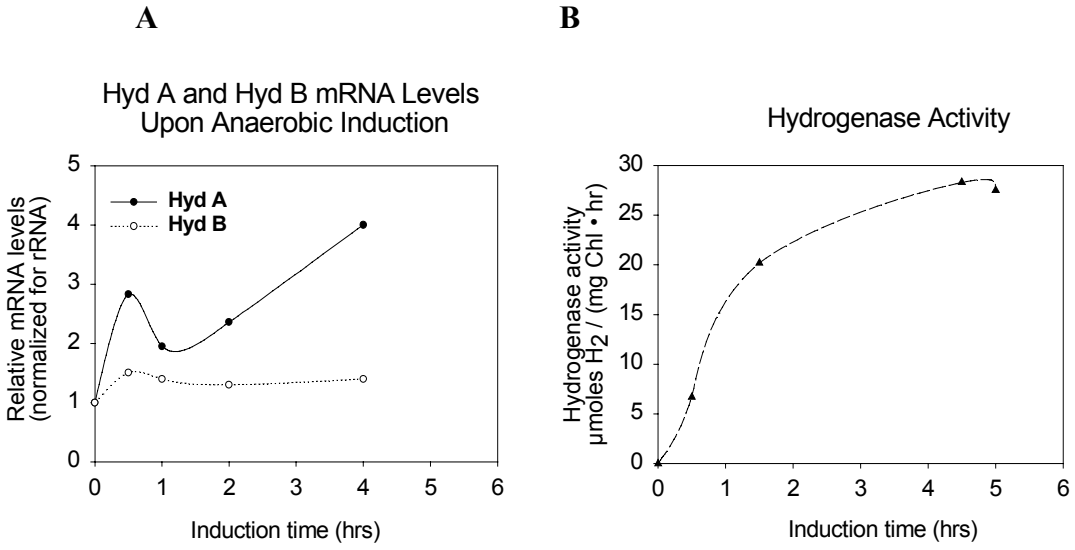

Figure 3. Quantitation of mRNA levels of Hyd A and Hyd B in *C. reinhardtii* cells (A) and overall enzyme activity as determined by amperometric measurement of H_2 production (B) upon anaerobic induction.

Previous work from our laboratory, using random chemical mutagenesis, resulted in the isolation of two sequential generations of H_2-producing mutants with increased tolerance to O_2 (Seibert et al. 2001). The loci affected by each mutation could not be determined, however, due to the lack of knowledge about the hydrogenase gene sequence. The availability of two algal hydrogenase clones will allow us now to map the above mutations and to start mutagenesis experiments in order to specifically address the O_2-tolerance problem associated with algal hydrogenases.

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