Footprint Analysis of the *psbAIII* Promoter Region in *Synechococcus* Strain sp. PCC 7942

Robert E. Garza
University Undergraduate Fellow, 1990-1991
Texas A&M University
Department of Biology

APPROVED:

Fellows Advisor Susan J. Golden

Honors Program Director

INTRODUCTION

Cyanobacteria perform oxygenic photosynthesis using a two-photosystem photosynthetic apparatus. This apparatus is similar to the thylakoid membrane system of higher plant chloroplasts (1). Photosystem II (PSII) is the complex at which H_2O is split, liberating O_2. The PSII reaction center consists of the following: 47 and 43 kilodalton (kDa) chlorophyll binding polypeptides, D1 and D2 polypeptides of approximately 32 kDa and cytochrome b-559 (2). D1 is encoded by the *psbA* gene which is highly conserved among plants and cyanobacteria. Higher plants contain a single copy of the *psbA* gene in the chloroplast genome (3,4), while cyanobacteria have multigene *psbA* families (5,6).

Synechococcus strain sp. PCC 7942, a cyanobacterium is useful for studying photosynthesis because it is highly transformable, i. e. it can take up and incorporate exogeneous DNA (8). This is an important feature for using recombinant DNA methods to study gene expression. *Synechococcus* contains three different copies of *psbA*, encoding two forms of D1 (7,8). It has been shown that each gene is capable of producing sufficient functional D1 to support photosynthesis in an actively growing culture (8). Form I, the product of *psbAI*, differs from Form II, the product of *psbAII* and *psbAIII*, at 25 of 360 amino acid positions. Twelve of these positions are in the first 16 amino acids of the protein.

Gene fusion experiments (9) have been done to monitor the expression of the *psbA* genes in *Synechococcus* at different light intensities. Translational fusions between each of the *Synechococcus psbA* genes and the *Escherichia coli lacZ* gene were inserted into the chromosome of wild-type *Synechococcus* at the respective *psbA* loci to serve as in vivo reporters of *psbA* expression. β-Galactosidase (product of *lacZ*) activities indicated differential expression of the *psbA-lacZ* gene fusions related to light availability. Expression of *psbAI* was greater than the expression of

psbAII and *psbAIII* under similar conditions. As light intensity decreased, expression of the *psbAI* reporter increased, whereas expression of the *psbAII* and *psbAIII* reporters decreased. In another study (10), Northern blot analysis was used to measure the levels of the *Synechococcus psbA* genes in a series of light shift experiments. After 15 minutes of exposure to high light intensities, the levels of a 1.2 kilobase (kb) *psbAII* transcript and the *psbAIII* transcript increased, while the *psbAI* transcript levels responded oppositely. There was also a 1.6 kb *psbAII* transcript which was unaffected by the changes in light intensity. In each of these studies the expression of the *psbA* genes was measured by mRNA levels or the levels of a reporter enzyme produced from gene fusions. These data, taken together, indicate that transcriptional regulation plays an important role in the light regulated expression of the *psbA* genes. This study focuses on the transcriptional regulation of the *psbAIII* gene in *Synechococcus*.

There are many examples, in prokaryotes and eukaryotes, of transcriptional regulation by proteins binding upstream of the open reading frame of a gene (11). These regulatory proteins act to activate or repress the transcription of the regulated gene. The binding sites are usually located in the promoter region or further upstream.

DNA mobility shift assays were done to determine if factors in *Synechococcus* protein extracts bound to the upstream region of *psbAIII*. The assays were repeated with smaller fragments to define the general binding area. A 190 bp fragment corresponding to a region flanking *psbAIII* formed DNA-protein complexes with *Synechococcus* protein(s). This fragment includes the promoter, approximately 50 additional upstream bases, the translational start site, and about 100 bases of the coding region of *psbAIII*. The binding reaction was optimized, and copper-phenanthroline DNA footprinting was done to identify the bases involved in the binding reaction.

MATERIALS AND METHODS

Strains

Synechococcus sp. used for extracts were grown in BG-11 liquid medium (12).

Escherichia coli strain DH5α *mcr-* , purchased from Bethesda Research Laboratories, Gaithersburg, Md., was the host for plasmids. Ampicillin (100 $\mu g \cdot ml^{-1}$) was added to LB growth medium (GIBCO Diagnostics, Madison, Wis.) (13) for selection of plasmids in *E. coli*.

DNA probe methods

Restriction enzymes, T4 DNA ligase, and Klenow polymerase were purchased from Boehringer Mannheim Chemicals, Indianapolis, Ind. DNA procedures including restriction digests, agarose gel electrophoresis, ligation and phenol-chloroform extraction, and 5'-endlabeling of DNA fragments with [α^{32}P] dCTP were carried out as described by Maniatis *et al.* (13).

Plasmids

Plasmid pAM676 contains a 450 bp blunt-ended *Bam*HI/*Xba*I fragment, including the promoter, about 280 additional upstream bases, the translational start site, and approximately 100 bases of coding region of the *psbAIII gene*, inserted into a *Sma*I cleaved Bluescript M13- (Stratagene). This plasmid was cloned by Ulrich Mueller.

Plasmid pAM912 contains a 250 bp blunt-ended *Hin*dIII/*Hpa*II fragment from pAM676 inserted into a *Sma*I cleaved Bluescript M13- (Stratagene). I cloned this plasmid.

Synechococcus protein extracts

For high light extract, the cells were shifted to 330 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 30 minutes

before harvesting. For the preparation of low light extracts, cells were grown at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ without shifting light intensity. Cells were harvested by centrifugation at 15,000*g* for 5 minutes. The pelleted cells were suspended with 15 ml of homogenization medium [50 mM Tris chloride (pH 7.5), 1 mM EDTA (pH 7.5), 2 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v)] and homogenized with an equal volume of glass beads (0.10-mm diameter) for 2 minute at 0 to 4°C in a homogenizing mill (Braun). The lysate was centrifuged and the supernatant was fractionated by loading onto a 3-ml heparin-Sepharose CL6B (Pharmacia LKB Biotechnology Inc., Piscatway, N. J.) column, equilibrated with 0.1M ammonium sulfate. The column was eluted with a 44-ml linear gradient of 0.1 to 1.0 M ammonium sulfate. Samples (10 µl) of each column fraction were assayed for DNA-binding activity by mobility shift assays. A more detailed account of these procedures is given by Chastain, *et al.* (14).

DNA mobility shift assays

Binding of factors in *Synechococcus* protein extracts to labeled *psbAIII* fragments was assayed by the gel electrophoresis mobility shift method (15). The 32µl binding reaction mixture contained the following: 5000 CPM of labeled fragment, 15µl of protein extract, 0.5 µg of poly dI-dC, 12% glycerol (v/v), 4mM Tris, 12mM Hepes, 60mM KCl, 0.5mM EDTA (pH 7.5). Poly dI-dC is a DNA copolymer that binds nonspecific DNA-binding proteins. The mixture was incubated at room temperature for 20 minutes. After this time, 3.2µl of loading buffer (10mM Tris-HCl pH 7.5, 50% glycerol (v/v), 0.01% bromophenol blue, 0.01% xylene cyanol) was added. The sample was loaded onto a 5% polyacrylamide gel and subjected to electrophoresis at 200V in Tris-glycine buffer (16). Once the bromophenol blue had run to the bottom, the gel was vacuum dried onto filter paper and exposed to X-ray

film overnight at room temperature.

Copper-phenanthroline footprinting

The following reagents were purchased from Sigma: 1,10-phenanthroline (OP); 2,9-dimethyl-1,10-phenanthroline (DM-OP); cupric sulfate; 3-mercaptopropionic acid (MPA). The copper-phenanthroline footprinting was done as described by Kuwabara and Sigman (17). A 56 ml binding reaction mixture included 60,000 CPM of endlabeled fragment, 48 µl of low light extract, 0.5 µg of poly dI-dC, 12% glycerol (v/v), 4 mM Tris, 12 mM Hepes, 60 mM KCl, and 0.5 mM EDTA (pH 7.5). The sample was subjected to electrophoresis on a 5% acrylamide gel as described for the DNA mobility shift assays. A sample with 48 µl of water instead of extract was also run to collect free probe. During the electrophoresis, solutions for the cleaving reaction were made. Solution A was prepared in the following manner: mixed equal volumes of 40 mM OP (in 100% ethanol) and 9.0 mM $CuSO_4$ (in water), and diluted 1/10 with water. Solution B contains 58 mM MPA in water. After electrophoresis the gel was immersed in 100 ml of 10 mM Tris (pH 7.5) and removed from the glass plate. 10 ml of solution A and 10 ml of solution B were added and the digestion was allowed to proceed for 15 minutes. The reaction was stopped by adding 10 ml of 28 mM DM-OP. This solution was allowed to stand for 2 minutes. The gel was rinsed with distilled water and exposed to X-ray film at room temperature for 3 hours. Bands of interest were cut from the gel, using the autoradiograph as a guide, and eluted overnight in TBE buffer (16). The eluted DNA was ethanol precipitated with yeast tRNA and resuspended in a urea-sucrose sequencing dye (16). The free and bound DNA were resolved on a 6% sequencing gel and measured against A+G, G, C, and C+T sequencing ladder markers (18).

RESULTS

Through mobility shift assays, using heparin-Sepharose column fractions, I detected a protein-DNA complex on a 415 bp *Hin*dIII/*Xba*I fragment containing about 100 bp of the *psbAIII* coding region and approximately 315 bp of upstream regions (Figure 1). The *psbAIII* promoter region does not form complexes with the same column fraction that binds to the *psbAI* and *psbAII* promoter regions. The assays were done using low light and high light extracts. Only the low light extract gave consistent binding. Subsequent experiments were done using low light extracts.

This fragment was cut into a 190 bp fragment, containing about 100 bp of *psbAIII* and 90 bp of the upstream region, and a 175 bp fragment with additional upstream bases. Mobility shift assays showed that only the 190 bp fragment formed a protein-DNA complex (data not shown). The two probes which formed a complex are diagrammed in figure 1. Figure 2 is a mobility shift assay of the 190 bp *psbAIII* fragment using high light and low light protein extracts. This fragment is actually a 250 bp *Xba*I/*Apa*I fragment when the bases from the multiple cloning site of the Bluescript M13- derived plasmid are included. The multiple cloning site contains a number of restriction sites. Since the *psbAIII* fragment was cloned into a *Sma*I site near the middle of the multiple cloning site and the *Xba*I and *Apa*I sites are close to the ends of it, extra bases are added to each side of the fragment. The nature of these restriction sites facilitates labeling the fragment on one end. This is important for footprint analysis as will be described later.

I did one experiment to try to optimize binding prior to the footprint analysis. Mobility shift assays were done in the presence or absence of 1 mM DTT and 2 mM Mg^{2+}. No difference in binding was observed for these different conditions (+DTT, +Mg^{2+}; +DTT, -Mg^{2+}; -DTT, + Mg^{2+}; -DTT, -Mg^{2+}), so subsequent experiments were done in the absence of DTT and Mg^{2+}.

Copper-phenanthroline is a chemical which cleaves DNA at different major groove sites to generate a series of DNA fragments. If a protein is bound to a particular sequence, this region will be protected from cleavage and will not generate bands when separated on a sequencing gel. A "footprint", or region of protein-dependent protection relative to a similarly treated fragment of free DNA, will be observed. The nucleotides involved in the protection can be determined exactly by running a sequencing ladder alongside the footprinting reactions. Some protein-DNA interactions facilitate the cleavage reaction. In this case the band on the bound DNA is stronger than the corresponding band for the free DNA. These are called hypersensitive sites.

The 250 bp *Xba*I/*Apa*I fragment was used for footprint analysis. The copper-phenanthroline method was chosen because of the apparent low concentration of binding proteins in the extract. If the concentration of the DNA binding protein is low, then a low percentage of the probe will be protected resulting in a high background of unprotected bands on the gel. The concentration problem is solved because the bound and free DNA segments are separated through polyacrylamide gel electrophoresis before treatment with the cleaving reagent. Figure 3 shows the footprint result. There are three protected regions which can be seen, and all are downstream of the transcription start site. With the transcription start site designated as +1, these regions are located at the following sites: +3 to +11, +16 to +27, and +35 to +41 (Figure 4). A hypersensitive site is present in two of the protected regions, and there are two hypersensitive sites upstream of the -35 region. More specifically, hypersensitive sites are located at -60, -41, +4, and +18.

DISCUSSION

The binding of regulatory proteins has rarely been observed within the transcribed sequence of the regulated gene. Footprint analysis of the *psbAII* promoter region has revealed two binding sites, both of which are present in the

transcribed sequence (U. W. Mueller and S. S. Golden, unpublished data). One site is an 11 bp direct repeat at the ATG start codon and a second site is centered around base +20. The footprints are separated by 6 bases, and appear to span approximately 25 bases. The data from the footprint of *psbAIII* indicates that it also has protein binding sites in transcribed sequences.

Testing of *Synechococcus* protein extracts with DNA mobility shift assays shows that the *psbAIII* promoter region does not form complexes with the same column fraction as the *psbAII* promoter region. Experiments done in Dr. Susan S. Golden's lab have shown that the *psbAIII* promoter region does not compete with the *psbAII* complexes (U. W. Mueller and S. S. Golden, unpublished data). These data indicate that a different factor binds *psbAIII*.

Low light extracts bind the *psbAIII* fragment stronger than high light extracts. This could be due to a higher protein concentration in the low light extract. A more interesting explanation could be that the binding protein is a repressor that is modified at high light levels, so that it has a lower binding affinity. This is only speculation. The protein concentrations of the two extracts need to be quantified, so that the extracts can be loaded equally.

The footprint must be improved before the protected and hypersensitive sites can be known with confidence. Once a clear footprint has been done, the next step is to determine which sites are necessary for binding. Mutations will be made in the fragment by site-directed mutagenesis. If a particular base is crucial to complex formation, then the complex will not be formed when that base is removed or mutated. The different upstream sequences generated can be fused to a reporter gene. The expression of the gene can be monitored to see if different upstream sequences affect transcription of the gene. If the absence of the protein-DNA complex can be correlated with decreased or increased transcription, then the protein is involved in transcriptional regulation of the *psbAIII* gene.

REFERENCES

1. Ho, K. K., and Krogmann, D. W. (1982) in *The Biology of Cyanobacteria* (Carr, N.G., and Whitton, B. A., eds) pp. 191-214, Blackwell Scientific Publications, Oxford
2. Satoh, K. (1985) *Photochem. Photobiol.* **42**, 845-853
3. Zurawski, G., Bohnert, H. J., Whitfeld, P. R., and Bottomley, W. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 7699-7703
4. Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. (1986) *Nature* **322**, 572-574.
5. Curtis, S. E., and Haselkorn, R. (1984) *Plant Mol. Biol.* **3**, 249-258
6. Gingrich, J. C., Buzby, J. S., Stirewalt, V. L., and Bryant, D. A. (1988) *Photosynth. Res.* **16**, 83-99
7. Schaefer, M. R., and Golden, S. S., (1989) *J. Biol. Chem.* **264**, 7412-7417
8. Golden, S. S., Brusslan, J., and Haselkorn, R. (1986) *EMBO J.* **5**, 2789-2798
9. Schaefer, M. R., and Golden, S. S. (1989) *J. Bacteriol.* **171**, 3973-3981
10. Bustos, S. A., Schaefer, M. R., and Golden, S. S. (1990) *J. Bacteriol.* **172**, 1998-2004
11. Ptashne, M. (1986) *Nature* **322**, 697-701
12. Allen, M. M., (1968) *J. Phycol.* **4**, 1-4
13. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Chastain, C. J., Brusca, J. S., Ramasubramanian, T. S., Wei, T. -F. and Golden, J. W. (1990) *J. Bacteriol.* **172**, 5044-5051
15. Fried, M., and Crothers, D. M. (1981) *Nucleic Acids Res.* **9**, 6505-6525
16. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) in *Current Protocols in Molecular Biology*, Wiley-Interscience, New York

17. Kuwabara, M. D., and Sigman, D. S. (1987) *Biochem. J.* **26**, 7234-7238

18. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* **65**, 499-559

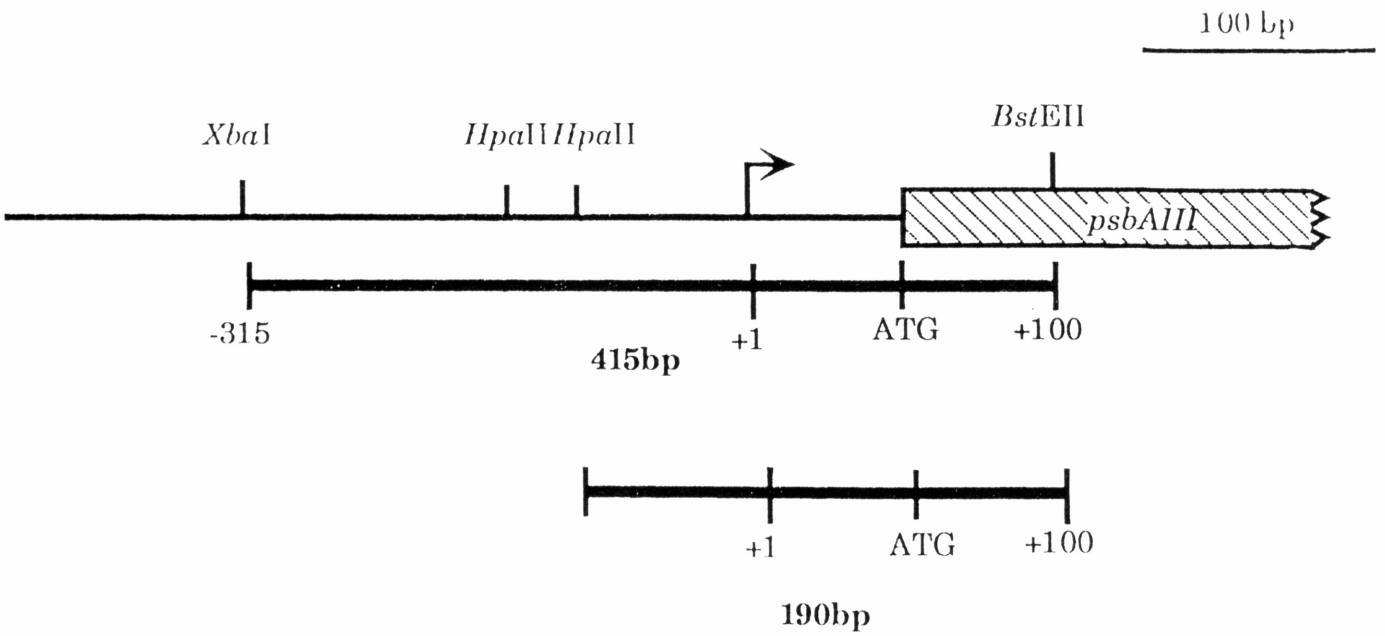

Figure 1. DNA probes used to analyze the binding of *Synechococcus* protein(s) to the promoter region of the *psbAIII* gene. The 415 bp probe is cloned into plasmid pAM676. The 190 bp probe is cloned into plasmid pAM912. The transcriptional start site is designated +1. The ATG site for the open reading frame of *psbAIII* is marked.

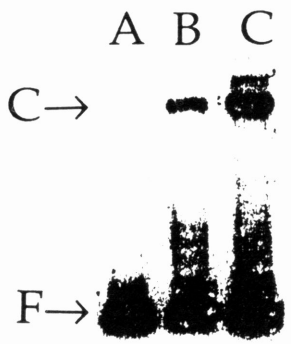

Figure 2. DNA mobility shift assay. A 190 bp fragment containing the promoter, about 50 additional upstream bases, the translational start site, and approximately 100 bases of the coding region for the *psbAIII* gene, was tested for binding to factors in *Synechococcus* protein extracts. The extracts were partially purified for DNA-binding proteins by heparin-Sepharose column chromatography. Lane A was loaded with the *psbAIII probe*. Lane B was loaded with the *psbAIII* probe and *Synechococcus* high light protein extract. Lane C was loaded with the *psbAIII* probe and *Synechococcus* low light protein extract. DNA-protein complexes (C) are present in lane B and lane C. Band F is the free probe.

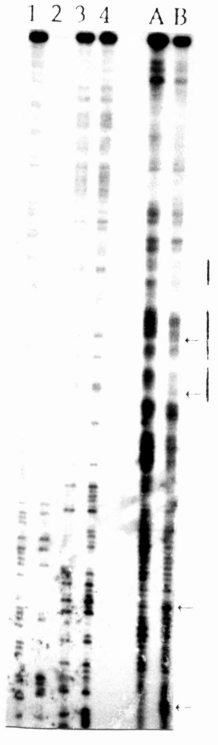

Figure 3. DNA footprint analysis of the *psbAIII* promoter region in *Synechococcus*. Lane A was loaded with the 190 bp *psbAIII* probe. This probe is described in Figure 1. Lane B was loaded with the probe plus a *Synechococcus* protein extract enriched for DNA binding proteins by heparin-Sepharose column chromatography. Lanes 1-4 were loaded with A+G, G, C, and C+T sequencing ladders. Protected regions are marked with vertical lines. Hypersensitive sites are marked by arrows.

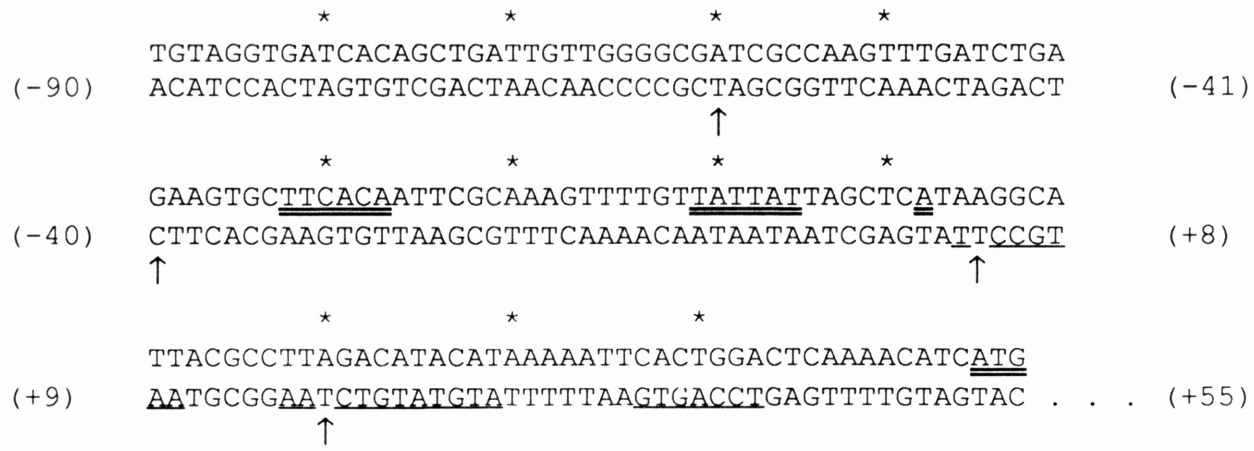

Figure 4. DNA sequence of the *psbAIII* promoter region of *Synechococcus* Strain sp. PCC 7942. The complementary strand for 100 bases of the *psbAIII* transcript and about 90 upstream bases was used to do the footprint. Positions are numbered with reference to the transcription start site. Protected regions and hypersensitive sites detected by footprint analysis are shown. Protected regions are underlined and hypersensitive sites are marked by arrows. The -35 region, -10 region, the translational start site, and the ATG site are double underlined. The asterisks mark 10 basepair intervals.