

Specific Binding of *Synechococcus* sp. Strain PCC 7942 Proteins to the Enhancer Element of *psbAII* Required for High-Light-Induced Expression

RIXIN LI, NAOMA S. DICKERSON,[†] ULRICH W. MUELLER,[‡] AND SUSAN S. GOLDEN*

Department of Biology, Texas A&M University, College Station, Texas 77843-3258

Received 18 August 1994/Accepted 15 November 1994

The *psbAII* gene of the cyanobacterium *Synechococcus* sp. strain PCC 7942 is a member of a three-gene family that encodes the D1 protein of the photosystem II reaction center. Transcription of *psbAII* is rapidly induced when the light intensity reaching the culture increases from 125 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (low light) to 750 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (high light). The DNA segment upstream of *psbAII* that corresponds to the untranslated leader of its major transcript has enhancer activity and confers high-light induction. We show that one or more soluble proteins from PCC 7942 specifically bind to this region of *psbAII* (designated the enhancer element). In vivo footprinting showed protein binding to the enhancer element in high-light-exposed cell samples but not in those maintained at low light, even though in vitro mobility shifts were detectable with extracts from low- or high-light-grown cells. When 12 bp were deleted from the *psbAII* enhancer element, protein binding was impaired and high-light induction of both transcriptional and translational *psbAII-lacZ* reporters was significantly reduced. This finding indicates that protein binding to this region is required for high-light induction of *psbAII*. The mutant element also showed impaired enhancer activity when combined with a heterologous promoter.

Light is an essential nutritional substrate for the photosynthetic cyanobacteria, and these organisms have evolved mechanisms to monitor the status of light in their environment. The existence of light-responsive signal transduction pathways in cyanobacteria is evident in the regulation of photosynthesis-related genes by both light quality (12, 33, 34) and light quantity (10). In the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, the photosystem II reaction center proteins D1 and D2 are encoded by *psbA* and *psbD* multigene families. When illumination of a PCC 7942 cell culture is increased, e.g., from standard growth conditions of 125 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (low light) to approximately 500 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (high light), four of five members of these two gene families are differentially expressed; only *psbDI* expression is unaffected by changes in light intensity. The expression of *psbAII*, *psbAIII*, and *psbDII* is rapidly induced by high light intensity, but expression of *psbAI* transiently decreases upon a shift to high light (2, 3, 15). All of the high-light-induced genes are regulated at the transcriptional level, and *psbAI* and *psbAIII* are also subject to post-transcriptional control (16).

Three distinct *cis* regulatory elements are present in the upstream regions of the high-light-induced *psbAII* and *psbAIII* genes: basal promoters which resemble *Escherichia coli* σ^{70} -type promoter sequences; negative elements upstream of the promoters; and light-responsive elements downstream of the promoters, within the untranslated leader regions of the *psbAII* and *psbAIII* mRNAs (19). The sequences downstream of the transcriptional start sites also have enhancer activity. PCC 7942 protein extracts enriched for DNA-binding proteins bind

specifically to the untranslated leader region of *psbDII* in vitro, and this binding was suggested to be important for *psbDII* expression (2).

We are interested in determining whether specific proteins that bind to the *psbAII cis* DNA elements contribute to the light-responsive regulation of *psbAII* expression through DNA-protein and/or protein-protein interactions. Here we show that partially purified PCC 7942 protein extracts specifically bind to a region of *psbAII* downstream of the transcriptional start site, which contains the enhancer element and light-responsive regulatory information, and form at least two distinct DNA-protein complexes. In vivo DNA footprinting demonstrated that specific protein binding to the untranslated leader region of *psbAII* occurs only in cells that have been subjected to high light intensity. A 12-bp deletion within the binding sites eliminated one of the two DNA-protein complexes and significantly reduced the induction of *psbAII* by high light. These results indicate that the interaction between *cis* and *trans* regulatory elements is required for high-light-induced expression of the *psbAII* gene in PCC 7942.

MATERIALS AND METHODS

Plasmids, strains, and culture conditions. Plasmids and strains are described in Table 1. Wild-type *Synechococcus* sp. strain PCC 7942 was grown in modified liquid BG-11 medium as described previously (2). Spectinomycin (20 $\mu\text{g}/\text{ml}$) was added to the medium for selection of recombinant strains. For controlled light shift experiments, cyanobacterial culture conditions, sample preparation, and β -galactosidase activity assays were as described previously (19).

Protein extracts and in vivo footprinting experiments used PCC 7942 or AMC052 cells that were cultured in a turbidostat under a standard light intensity of 125 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (hereafter referred to as low light) and at an optical density at 750 nm of 0.6 to 0.8. High-light samples were collected after the culture was exposed to 550 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 30 min in the turbidostat.

E. coli strains were grown in standard LB medium (24), with addition of the antibiotics ampicillin (100 $\mu\text{g}/\text{ml}$), spectinomycin (50 $\mu\text{g}/\text{ml}$), and chloramphenicol (17 $\mu\text{g}/\text{ml}$) as needed for plasmid selection.

Partial purification of DNA-binding proteins. One liter of high-light-exposed PCC 7942 cells was harvested from the turbidostat and collected by centrifugation at 4,000 $\times g$ for 10 min. Soluble protein extracts were prepared from the sample and enriched for DNA-binding proteins by using heparin-Sepharose column chromatography as described by Chastain et al. (6). Column fractions

* Corresponding author. Mailing address: Dept. of Biology, Texas A&M University, College Station, TX 77843-3258. Phone: (409) 845-9824. Fax: (409) 845-2891. Electronic mail address: SGOLDEN@TAMU.EDU.

[†] Present address: Department of Biological Sciences, Clemson University, Clemson, SC 29634-1903.

[‡] Present address: Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
<i>Synechococcus</i> strains		
PCC 7942	Wild type	Laboratory collection
AMC052	<i>psbAII-lacZ</i> reporter strain used for in vitro footprinting	26
AMC153	<i>psbAII-lacZ</i> reporter gene fusion integrated at a neutral site in the PCC 7942 chromosome; upstream of the reporter gene is the -70 to +110 region of <i>psbAII</i>	This study
AMC154	<i>psbAII-lacZ</i> reporter gene fusion integrated at a neutral site in the PCC 7942 chromosome; upstream of the reporter gene is the -70 to +100 region of <i>psbAII</i> with a 5-bp deletion in the untranslated leader sequence	This study
AMC295	<i>psbAII-lacZ</i> reporter gene fusion integrated at a neutral site in the PCC 7942 chromosome; upstream of the reporter gene is the -70 to +100 region of <i>psbAII</i> with a 12-bp deletion in the untranslated leader sequence	This study
<i>E. coli</i> strains		
DH10B	<i>mcrA mcrB</i> , host strain for transformation	Bethesda Research Laboratories
MC1061	<i>hsdR Δlac X74</i> ; used for site-directed mutagenesis	4
CJ236	<i>dut ung F'</i> ; used for site-directed mutagenesis	17
Plasmids		
pBluescript KS+	Cloning vector, encodes ampicillin resistance	Stratagene
pBluescript II KS+	Cloning vector, encodes ampicillin resistance	Stratagene
pMC1871	Source of truncated <i>lacZ</i> gene for translational fusions	29
pAM697	Source of -242 to +192 region of <i>psbDI</i> used as competitor DNA in mobility shift assays	2
pAM737	Source of -96 to +162 region of <i>psbDII</i> used as competitor DNA in mobility shift assays	2
pAM747	-70 to +110 of <i>psbAII</i> cloned as a <i>SmaI-HindIII</i> fragment in pBluescript KS+	This study
pAM854	PCC 7942 recombination vector; contains a spectinomycin resistance cassette in a PCC 7942 neutral site	2
pAM912	-165 to +113 region of <i>psbAIII</i> cloned in pBluescript KS+; the insert was used as competitor DNA in mobility shift assays	This study
pAM980	Similar to pAM747 except 5 bp of <i>psbAII</i> from +20 to +24 were deleted by site-directed mutagenesis	This study
pAM991	Source of <i>psbAII</i> open reading frame internal fragment (+702 to +928) used as competitor DNA in mobility shift assays	21a
pAM1003	pAM854 with the -70 to +110 region of <i>psbAII</i> fused in frame with a truncated <i>E. coli lacZ</i> gene; PCC 7942 recombination substrate for AMC153	This study
pAM1005	Similar to pAM1003 but has 5-bp-deleted <i>psbAII</i> from +20 to +24; PCC 7942 recombination substrate for AMC154	This study
pAM1229	Similar to pAM980 except an additional 7 bp (+26 to +32) of <i>psbAII</i> were deleted	This study
pAM1325	+1 to +41 region of <i>psbAII</i> cloned into the <i>EcoRV</i> site of pBluescript II KS+	This study
pAM1369	Similar to pAM1005 but has an additional 7 bp (+26 to +32) deleted in <i>psbAII</i> ; PCC 7942 recombination substrate for AMC295	This study

were surveyed for activity while in column buffer (Fig. 1A) but were dialyzed (6) prior to subsequent mobility shift assays (e.g., Fig. 1B). To further purify the DNA-binding proteins for some experiments including in vitro footprinting, two or three enriched fractions containing specific DNA-binding activity were combined and loaded onto a 1-ml heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) column equilibrated with 0.1 M ammonium sulfate in buffer A (50 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 0.1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) (6). The column was washed with 10 ml of 0.1 M ammonium sulfate in buffer A and eluted with 1.5 ml steps of 0.2, 0.3, 0.4, and 0.5 M ammonium sulfate in buffer A. The 0.3 M fraction contained most of the binding activity, and the proteins from this fraction were further concentrated by centrifugation at 5,000 × g for 1 h, using a 30K Microsep concentrator (Filtron Technology Co., Northborough, Mass.).

Preparation of DNA fragments for mobility shift assays. DNA fragments from pAM747, pAM980, pAM1229, and pAM1325 were released for labeling by digestion with *XbaI* and *HindIII*. Each fragment was labeled with [α -³²P]dCTP, using the Klenow fragment of DNA polymerase I (1). Other DNA fragments used as probes in mobility shift assays were obtained by PCR, using pAM747 as the template and an appropriate pair of primers corresponding to the ends of each fragment. The labeled PCR products were gel purified, and the eluted DNA fragments were used in mobility shift assays. DNA fragments used as competitors in the assay were either released from appropriate plasmids (Table 1) with restriction enzymes or generated by PCR.

DNA mobility shift assays. Assays were performed as described by Ausubel et al. (1) under conditions of protein excess such that more than 50% of the labeled

probe was bound. A sample of heparin-Sepharose-fractionated proteins (4 to 10 μ g) was incubated with 0.1 to 0.5 ng of labeled DNA fragment in binding buffer (4 mM Tris-HCl [pH 8.0], 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 12% glycerol, 5 mM MgCl₂, 60 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol) containing 0.5 to 1 μ g of poly(dI-dC) in a final volume of 20 μ l. After 20 min of incubation at room temperature, the samples were loaded onto a 5% polyacrylamide gel (10 by 7.5 cm; acrylamide/bisacrylamide, 30:1; 50 mM Tris, 380 mM glycine, 2 mM EDTA, 2.5% glycerol) and subjected to electrophoresis at 150 V for 20 to 40 min in the running buffer (50 mM Tris [pH 8.5], 100 mM glycine, 2 mM EDTA). Gels were dried and exposed to X-ray film.

DNA footprinting. In vitro footprinting was performed by using the 1,10-phenanthroline copper (Cu-OP) reagent described by Kuwabara and Sigman (18). The DNA insert was released from pAM747 (Table 1) by digestion with *XbaI* and *KpnI*, and the fragment was labeled at the *XbaI* site through incorporation of [α -³²P]dCTP by the Klenow enzyme (1). A DNA sample (40,000 cpm) was incubated with 30 μ l of protein extract (25 μ g of total protein) for mobility shift assay. Cu-OP cleavage, excision of labeled DNA bands, and subsequent electrophoresis were performed as previously described (2).

In vivo footprinting was performed as described by Sasse-Dwight and Gralla (25), with the following modifications. A sample of AMC052 culture (750 ml) that was maintained under low light or was exposed for 30 min to high light was transferred from the turbidostat to a 1-liter glass cylinder, illuminated the same as before the transfer, and stirred vigorously. Dimethyl sulfate (DMS) was added to each culture to a final concentration of 5 mM for 5 min. The reaction was stopped by chilling the samples rapidly with ice, and the cells were harvested by

centrifugation at $4,000 \times g$ for 10 min at 4°C . Parallel control samples of low- and high-light-grown cyanobacterial cells without DMS treatment were also harvested from the turbidostat. Chromosomal DNA was isolated from all the samples as previously described (3). DNA from two control samples was resuspended in 100 μl of DMS buffer (50 mM sodium cacodylate [pH 7.0], 1 mM EDTA) and treated with 6.7 μl of 150 mM DMS for 3 min at 37°C . The reaction was quenched with 200 μl of stop buffer (3 M ammonium acetate, 1 M 2-mercaptoethanol, 250 μg of tRNA per ml, 20 mM EDTA). All methylated DNA samples from both the in vivo and in vitro treatments were resuspended in 100 μl of 1 M piperidine and were cleaved at 90°C for 30 min. Samples were quenched briefly on ice and then spun through a 1-ml Sephadex G-50-80 (Sigma Chemical Co.) column preequilibrated in Tris-EDTA at $1,700 \times g$ for 2 min. Each DNA sample (20 μg) was analyzed by primer extension with a ^{32}P end-labeled specific oligonucleotide, using a thermal cycling protocol (25). After extraction and precipitation of the reaction products, the samples were separated on an 8% sequencing gel next to sequencing ladders generated by the same primer, an appropriate plasmid template, and the SequiTherm cycle sequencing kit (Epicenter Technologies, Madison, Wis.). The gel was dried and exposed to X-ray film at -80°C for 48 h.

Construction of *psbAII* binding site mutants. A 5-bp deletion extending from +20 to +24 relative to the *psbAII* transcription start site was made by the Kunkel method of site-directed mutagenesis as described by Ausubel et al. (1). The mutagenic oligonucleotide was 5' TATTAAGTCTCGTGTCACTAAG 3'. This deletion construct, designated delta 5 (pAM980), generated an *XhoI* restriction site. This *XhoI* site and an adjacent *DdeI* site were cleaved, and unpaired bases were filled in with the Klenow enzyme prior to ligation, deleting seven additional nucleotides (from +26 to +32) and creating the delta 12 mutation (pAM1229). Both deletions were confirmed by nucleotide sequence analysis. Translational *lacZ* fusions were constructed by using the wild-type and both mutant versions of the -70 to +110 segment of the *psbAII* sequence (ATG begins at +50) and a truncated *lacZ* reporter gene (29). These fusion constructs were cloned into pAM854, which contains a PCC 7942 neutral site and a spectinomycin resistance cassette (2). The resulting plasmids, pAM1003, pAM1005, and pAM1369, were used to transform PCC 7942 (11). Each fusion construct was integrated into the cyanobacterial genome upon recombination between the neutral site on each plasmid and the chromosome. The recombinant strains AMC153, AMC154, and AMC295 were confirmed by PCR using specific primers.

Computer-generated images. Graphic images were generated with an Apple OneScanner and Ofoto software (version 2.0; Light Source Computer Images, Inc.). Files were transferred to Canvas (version 3.0.6; Deneba Systems, Inc.) for lettering and printed on a Tektronix Phaser IISD dye-sublimation printer. The Cu-OP footprint was visualized by a Fujix BAS 2000 phosphorimaging system. Quantitation of bands from scanned autoradiograms was performed with NIH Image software (version 1.52).

RESULTS

Proteins from PCC 7942 cells bind specifically to the upstream region of *psbAII*. To determine whether putative regulatory proteins bind to the *psbAII* gene, a DNA probe that encompasses the promoter region of *psbAII* and extends into its open reading frame (-70 to +110 relative to the transcription start site) was used in DNA mobility shift assays. Soluble protein extracts from *Synechococcus* sp. strain PCC 7942 were enriched for DNA-binding proteins by heparin-Sepharose column chromatography and elution with a linear 0.1 to 1.0 M ammonium sulfate gradient. A single peak of DNA-binding activity, spanning several column fractions from both low- and high-light extracts, was detected by the probe (data not shown). Immunoblot analysis revealed that RNA polymerase core and sigma subunits elute with a profile different from the DNA-binding activity. These proteins were barely detectable in the most active DNA-binding fractions, and they peaked in fractions that showed no DNA-binding activity (data not shown). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1) indicated that the active DNA-binding fractions contain 20 to 30 bands detectable by silver staining (data not shown). Figure 1A shows a mobility shift assay using high-light extract fractions and the *psbAII* probe. The fraction showing peak binding activity (fraction 11) was used for subsequent analysis. The typical pattern of DNA-protein complex formation with this probe in response to increasing protein concentration is shown in Fig. 1B. At low protein concentrations, a single complex, C1, was formed. As the protein concentration increased, a second complex, C2, appeared. This complex could be resolved into

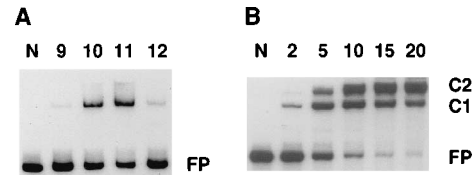


FIG. 1. DNA mobility shift assays of the *psbAII* upstream region with heparin-Sepharose fractions from a PCC 7942 cell lysate. (A) Column fractions marked by the number above each lane were tested for binding activity with a *psbAII* probe extending from -70 to +110 relative to the transcription start site. Five microliters of protein extract from each fraction was equilibrated with 3,000 cpm of labeled DNA fragment in binding buffer and analyzed on a 5% polyacrylamide gel. Lane N (no extract) contains only free probe, and its migration is indicated by FP. C1 and C2 represent the two DNA-protein complexes. (B) DNA-protein complex formation of the *psbAII* upstream region with increasing binding-competent protein extracts (fraction 11 from panel A, dialyzed). The amount of extract (in microliters) added to each reaction is shown above each lane.

more than one band and often ran as a doublet or a triplet. A further increase in the protein concentration increased the C2 level but decreased the amount of C1. At the highest protein concentrations used, the C1 complex disappeared and only C2 was observed (data not shown).

To determine whether protein binding to the *psbAII* probe was specific, unlabeled DNA fragments from different sources were used in competition assays as shown in Fig. 2. Self-competition analysis showed that a 25-fold molar excess of the unlabeled probe fragment eliminated formation of the C2 complex, and a 50-fold molar excess of the competitor reduced protein binding to the probe by 80% (Fig. 2A). An unlabeled fragment that is internal to the *psbAII* open reading frame (Fig. 2D) and a fragment that contains the promoter of the *psbDI* gene (Fig. 2E) did not compete well with the same probe; binding was decreased only 18 and 25%, respectively, by these fragments at 50-fold molar excess. Interestingly, when unlabeled *psbAIII* and *psbDII* upstream region fragments were used as competitors, formation of the C2 complex was signif-

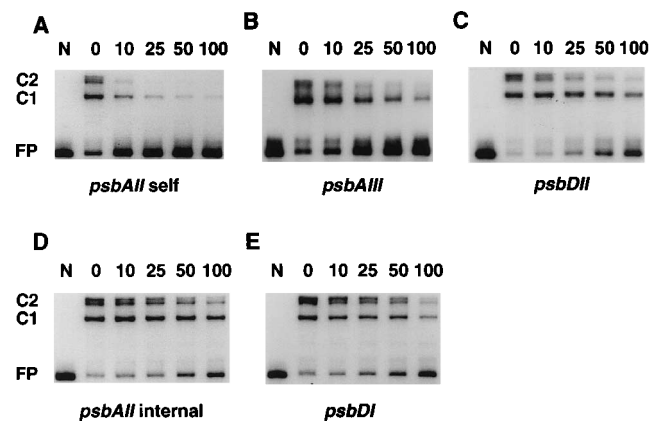


FIG. 2. Competition for *psbAII*-binding proteins by different specific and nonspecific DNA fragments. Unlabeled DNA fragments were tested for the ability to compete for protein binding to the *psbAII* upstream region (-70 to +110 labeled probe as in Fig. 1). Competitor fragments: (A) *psbAII* from -70 to +110 (self-competition); (B) *psbAIII* upstream sequence from -165 to +113; (C) *psbDII* upstream sequence from -96 to +162; (D) *psbAII* internal open reading frame sequence from +703 to +928; and (E) *psbDI* upstream sequence from -242 to +192. The molar excess of the competitor fragment is indicated above each lane. Protein extract (10 μl) was incubated with labeled *psbAII* probe in the presence or absence of competitor DNA; lane N in each panel lacks protein extract. FP, C1, and C2 indicate the migrations of free probe and two DNA-protein complexes, respectively.

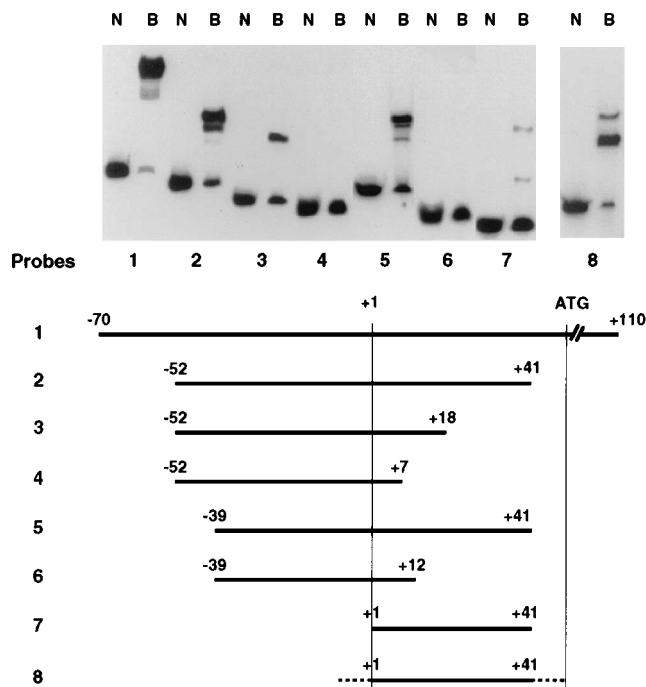


FIG. 3. Localization of the specific binding sites in the *psbAII* upstream region. DNA mobility shift assays were performed with protein extracts and labeled DNA fragments containing different regions of *psbAII* upstream sequence. The endpoints of each probe are indicated by nucleotide numbers relative to the *psbAII* transcription start site. The dashed lines flanking the *psbAII* sequence in probe 8 represent the multiple cloning site sequences from the pAM1325 vector. Samples in N lanes contain only free probe in the binding buffer; 10 μ l of protein extract was added to each B-lane sample. Samples containing probe 1, which is longer than the other probes, were loaded onto the gel and run for 10 min before the other samples were loaded for electrophoresis.

icantly inhibited at 25-fold molar excess, and at 50-fold molar excess, the total binding was decreased by 54 and 41%, respectively (Fig. 2B and C). These data showed that the binding of PCC 7942 protein(s) to the *psbAII* upstream region is specific and suggest that the same factor(s) binds to the upstream regions of *psbAIII* and *psbDII*.

Localization of the specific protein-binding sites on *psbAII*.

We wanted to localize the protein-binding sites of *psbAII* and determine whether they lie within one or more of the three *cis* regulatory elements identified previously (19). Mobility shift assays were performed with a series of PCR-generated probes containing different *cis* elements within the -70 to $+110$ region of *psbAII* sequence, and the results are shown in Fig. 3. The degree of binding and the pattern of the complexes formed with probe 2, which contains all three *psbAII* *cis* regulatory elements, are comparable with those properties of probe 1, indicating that this region accounts for all the binding sites present in probe 1. Probe 5, which eliminated the negative element upstream of probe 2, formed a similar pattern of complexes but showed a slightly lower binding capability than probe 2. Probe 3, which contains the negative element, basal promoter, and part of the enhancer element (-52 to $+18$), consistently formed only one DNA-protein complex, suggesting that one protein-binding site is present within this region, but another binding site requires sequences further downstream of $+18$ (compare the binding pattern of probe 3 with that of probe 2). When 11 bp (-52 to $+7$) were removed from the right end of probe 3 to create probe 4, the fragment did not show any binding activity. While the basal promoter alone

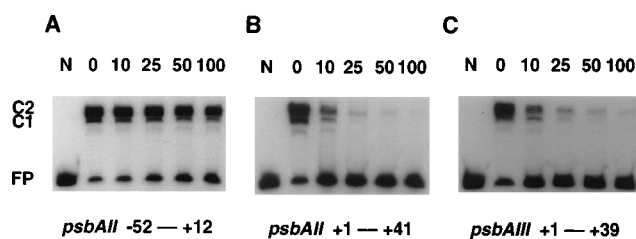


FIG. 4. Competition of *psbAII* and *psbAIII* *cis* regulatory elements for *psbAII*-binding proteins. Mobility shift assays were carried out with 10 μ l of twice-heparin-Sepharose-fractionated protein extract and 0.1 ng of labeled *psbAII* probe 2 (Fig. 3) with or without unlabeled competitor DNA fragments. Competitor DNA fragments: (A) the negative element and basal promoter of *psbAII* (-52 to $+12$); (B) the enhancer and high-light-responsive element of *psbAII* ($+1$ to $+41$); (C) the enhancer and high-light-responsive element of *psbAIII* ($+1$ to $+39$). The molar excess of competitor added to the reaction is indicated above each lane. The positions of free DNA probe and the two DNA-protein complexes are shown as FP, C1, and C2, respectively.

(probe 6) was unable to form any stable complex, the sequences downstream of the transcription start site containing the enhancer and light-responsive element (probe 7) formed two distinct but weak complexes. When this probe was flanked with 10 to 15 bp of nonspecific DNA at each end (probe 8), more prominent complexes were formed, and 100% binding could be achieved by addition of 30% more protein extract (data not shown).

To ensure that the binding of proteins downstream of the *psbAII* transcription start site is specific, competition assays were performed with a fragment that comprises all three known *cis* elements as the probe and fragments containing one or two of the *cis* elements as competitors (Fig. 4). The protein extracts used in this experiment and hereafter were twice heparin-Sepharose fractionated and microconcentrator treated (see Materials and Methods). It was clear that the negative element and basal promoter of *psbAII* (-52 to $+12$) were not sufficient to compete for binding with the probe, but the untranslated leader regions of both *psbAII* ($+1$ to $+41$) and *psbAIII* ($+1$ to $+39$) significantly competed for binding at 25-fold molar excess.

To directly locate the protein-binding sites, DNA footprinting using Cu-OP was performed with the upstream region of *psbAII*. Two protein-binding regions were localized, one extending from -4 to $+32$ and another around the ATG start codon from $+42$ to $+67$ (data not shown).

In vivo footprinting of the *psbAII*-binding site under low and high light intensities. We performed in vivo methylation protection studies under low- and high-light conditions to determine whether the binding of regulatory factors to the *psbAII* untranslated region differs under the two light regimens. If a region of DNA is bound by proteins, certain G, and sometimes A, residues that contact the protein(s) should remain unmodified by in vivo treatment with DMS. Piperidine-cleaved DNA isolated from the in vivo-treated samples can be compared with parallel samples in which isolated naked DNA was modified in vitro. Protected G or A residues in the in vivo-treated samples should indicate regions bound by protein(s) that may be important in gene regulation. We used primer extension with radiolabeled oligonucleotides to analyze both strands of the *psbAII* upstream region, specifically the binding sites identified by Cu-OP footprint and mobility shift assays. For this analysis, we used AMC052, a reporter strain that carries a *psbAIII-lacZ* translational fusion, rather than the wild-type strain. The reporter strain allowed us to examine the RNA-like strand (designated the upper strand) with a primer comple-

mentary to the *lacZ* portion of the reporter gene. This approach was necessary because the corresponding region of the native *psbAIII* gene is indistinguishable from the *psbAIII* gene, and a *psbAIII*-specific primer could not be generated. Comparison of the piperidine cleavage pattern of the DNA samples treated *in vitro* and *in vivo* with DMS showed that on the upper strand, three G residues (-18, +14, and +37) and three A residues (+1, +31, and +42) were protected in the high-light DMS-treated sample but not in the equivalent low-light sample (compare lanes 2 and 3 with lanes 5 and 6 in Fig. 5A). On the lower strand, one G (+16) and two A residues (+20 and +27) were also protected only in the high-light sample treated *in vivo* with DMS (Fig. 5B). We also observed two protected C residues, one in each strand, that were protected as G residues on the opposite strand (+16 and +37 in Fig. 5). This finding suggests that the protein factor(s) was bound to this region of *psbAIII* *in vivo* only under high-light conditions. The nucleotides identified by Cu-OP or by DMS *in vivo* footprinting are summarized in Fig. 5C.

Functional analysis of the *psbAIII*-binding-site deletion mutants. To assess the relationship between protein binding and light-responsive gene expression, we constructed *psbAIII*-binding-site deletion mutants and investigated their abilities to bind proteins *in vitro* and to drive expression of a reporter gene *in vivo*. Mutant delta 5 has a 5-bp deletion from +20 to +24 relative to the *psbAIII* transcription start site, and mutant delta 12 has an additional 7-bp deletion from +26 to +32 (see Materials and Methods). The protein-binding abilities of the mutant fragments were compared with that of the wild-type DNA in a mobility shift assay (Fig. 6). With the delta 5 mutant, we did not observe significant changes in its binding ability or pattern of complex formation in most experiments; with delta 12, however, we consistently obtained only one stable complex in the assay (Fig. 6), similar to the binding pattern of probe 3 in Fig. 3. This finding implied that one protein-binding site was removed in the delta 12 mutant.

The *in vivo* expression of the *psbAIII*-binding-site mutants was compared with that of the wild type in a controlled light shift experiment using translational *lacZ* fusion reporter genes integrated into the PCC 7942 chromosome at a neutral site. Figure 7A illustrates each of the gene fusions and shows the confirmation of the genotype of each reporter strain. PCR products were generated by using two pairs of specific primers and lysed cyanobacterial cells from each strain as the template. When primers 1 and 2 were used in PCR, two amplified products, one from the native chromosomal *psbAIII* locus and another from the reporter gene at the neutral site locus, were detected in each strain. This was best observed in the mutant strains in which the two PCR products can be distinguished on a 5% polyacrylamide gel: the deletion mutant copies of the amplified *psbAIII* region ran 5 and 12 bp lower than the native *psbAIII* PCR product in strains AMC154 and AMC295, respectively. When a third primer from the *lacZ* gene sequence was used together with primer 1, we observed one product of expected size from the *psbAIII-lacZ* fusion in each strain. The 5- and 12-bp differences of the bands in AMC154 and AMC295 were not resolved for this larger PCR product.

Culture samples from each reporter strains were collected 2 h before and at the time light was shifted from 125 to 750 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and 2 h after the light was shifted. β -Galactosidase specific activity was assayed from each sample. Figure 7B shows the combined results from three independent experiments. It was clear that while the low level of *psbAIII-lacZ* expression under low light was maintained in the wild-type and mutant strains, the induced expression under high light was reduced in both mutants in comparison with the wild type

(AMC153). For AMC154, in which the *lacZ* gene was driven by the delta 5 mutant control region, the induction of β -galactosidase activity after 2 h under high light was 5-fold, down from more than 10-fold in the wild type. In AMC295, the 12-bp-deleted *psbAIII-lacZ* strain, the high-light induction was less than twofold. We constructed transcriptional *lacZ* fusion in which the mutant *psbAIII* untranslated leader region from delta 12 was combined with an *E. coli conII* promoter. Enhancer activity of the mutant element was only 2.5-fold, compared with 11-fold in the corresponding wild-type construct, and no high-light induction was detectable for the mutant element (data not shown).

DISCUSSION

DNA mobility shift assays indicated that proteins from the same heparin-Sepharose-resolved fraction bind to the upstream regions of *psbAIII* and two other high-light-induced photosystem II gene family members, *psbDII* and *psbAIII*, and that they compete with each other for binding proteins (2, 21). The results of these competition assays are consistent with an earlier study which showed that when a *psbDII* upstream region is used as the probe, a *psbAIII* upstream sequence can compete for protein binding more efficiently than the unlabeled *psbDII* fragment itself. We conclude that one or more factors binds to both *psbAIII* and *psbDII*, but that the affinity for the *psbAIII*-binding sites is higher. However, *psbDII* also forms a DNA-protein complex that is not affected by the presence of excess *psbAIII* upstream DNA, suggesting binding of a factor that is not shared by the two genes (2). The upstream region of *psbDI*, whose expression is unaffected by light intensity, did not show binding ability and did not compete efficiently for *psbAIII*- and *psbDII*-binding proteins (Fig. 2E and reference 2). These results suggest that specific protein binding to the different gene sequences directly or indirectly influences the differential expression of the *psbA* and *psbD* gene families in response to changes in light intensity. We used mobility shift assays and *in vitro* Cu-OP footprinting to localize the protein-binding sites of *psbAIII* to regions downstream of the transcription start site, and particularly within the enhancer region that includes the light-responsive element (19). The fact that the *psbAIII* untranslated leader region effectively competed with the *psbAIII* fragment for protein binding (Fig. 4C) agrees with the *in vivo* study which showed that the two regions share the same functions (19). Examination of the *psbAIII* and *psbAIII* enhancers, and the previously identified *psbDII* protein-binding sites (2), did not reveal any notable sequence similarity among them or with other commonly known protein-binding sequences, yet the same protein(s) appears to bind to the untranslated leader region of the three genes. This observation supports a hypothesis that the DNA structure, rather than its linear sequence, may be important for protein recognition (2, 19). However, circular permutation assays (14) indicated that the enhancer region of *psbAIII* does not include an intrinsic bend, nor is it bent by protein binding (data not shown).

The physical locations of the protein-binding sites of *psbAIII* and *psbDII* (2) are in the transcribed but untranslated leader regions of each mRNA. Similar examples of this arrangement of regulatory elements were recently found in other bacterial species. The developmentally regulated *Caulobacter flaNQ* promoter is activated by an enhancer and integration host factor-binding elements, both located downstream of the transcription start site (9). In mucoid *Pseudomonas aeruginosa*, two of three AlgR1-binding sites in the osmolarity-responsive *algC* promoter region are downstream of the transcription start site: one in the untranslated leader region, and another inside the

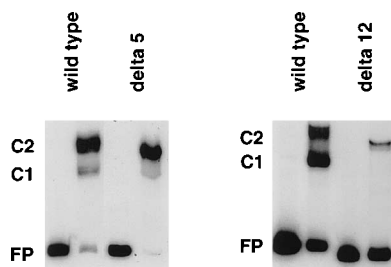


FIG. 6. Specific protein binding to wild-type and mutant *psbAII* upstream regions. DNA mobility shift assays were performed with 5 μ l of twice-heparin-Sepharose-fractionated protein extracts (5.5 μ g of total protein) incubated with labeled *psbAII* wild-type probe 1 (Fig. 3) and the equivalent fragment of delta 5 or delta 12. Samples were separated on a 5% polyacrylamide gel, and the migrations of free probe and two DNA-protein complexes are indicated as FP, C1, and C2, respectively.

means of gene regulation that can allow the organism to quickly respond to certain environmental stimuli.

The similar patterns of complexes formed by probes 1 and 2 (Fig. 3) suggest that a fragment which lacks *psbAII* open reading frame sequences accounts for all binding sites, but subtle differences may not be resolved by the mobility shift assay. Indeed, our Cu-OP footprinting also identified a protected region further downstream of the enhancer, around the translational start codon. However, this region used alone as a probe did not form specific DNA-protein complexes (21). It is possible that a factor(s) binds to this region only when sites within the enhancer are present and occupied; thus, the fragment would show no binding activity when tested alone. Alternatively, the probe fragment may not have been appropriate to allow stable binding. The enhancer element flanked by non-specific DNA sequence shows increased binding ability relative to the element alone, implying that the sequences adjacent to the element may help the binding protein(s) anchor on the specific sites (probe 8 versus probe 7 in Fig. 3). It is also possible that all sites necessary for protein binding are in the enhancer region, but that bound factors contact the translation start and leave a footprint in this region.

Our heparin-Sepharose chromatography did not resolve any factors that bind specifically to the negative *cis* element located upstream of the *psbAII* promoter. Changes to the protein purification or assay conditions, or an *in vivo* genetic selection strategy, may be required to identify a protein factor(s) that binds this element. It is also possible that the negative element does not act through a specific binding protein(s) but has an intrinsic functional property conferred by its nucleotide sequence.

In vivo DNA footprinting has been used by other researchers to study protein-DNA interaction in different organisms (25, 28). Schmidt-Goff and Federspiel showed that in another cyanobacterium, *Fremyella diplosiphon*, a region of DNA upstream of the light-quality-regulated *cpeBA* operon is bound by protein *in vivo* under both red and green light, although *cpeBA* expression is induced only under green light (27). In our early investigations of protein binding to *psbA* genes, we tested low- and high-light-exposed PCC 7942 cell extracts in our *in vitro* mobility shift assays and detected specific *psbAII*-binding activity in both (data not shown). Thus, we were interested in knowing whether proteins also bind to the same region of *psbAII* *in vivo* under both low and high light intensities, and subsequently, whether there is a relationship between specific protein binding and light-responsive gene expression. Our *in vivo* footprinting results showed that only under high light,

three G residues and five A residues within the untranslated leader region of *psbAII* were protected. This finding implies that the binding protein(s) of PCC 7942 undergoes specific modification when cells are subjected to high light intensity and that only one modification state of the protein(s) has the binding competency that is associated with induced gene expression under high light.

We have not established definitively whether the binding activities in low- and high-light extracts differ with respect to *in vitro* complex formation with DNA. Laboratories studying *in vitro* protein binding to two other cyanobacterial light-regulated genes, the green/red-regulated *cpeBA* (27, 31) and *cpc2* (5, 30) operons of *Calothrix* sp. strain PCC 7601 (*F. diplosiphon* Fd33), have been conflicting. For each regulatory region, one laboratory has reported binding activity in extracts from cells grown in only one of the two light regimens, and the other has shown binding activity in extracts from both. *In vitro* assays have the intrinsic problems of possible modifications to the protein during isolation and unnatural concentrations of DNA and protein that may mask subtle differences in binding affinities. We have therefore chosen to use *in vitro* assays as a means of identifying the protein-binding sites but *in vivo* assays as the major avenue for determining the function of the interaction.

Phosphorylation has been reported as a common way of protein modification. The bacterial enhancer-binding protein NtrC can bind to its target sites *in vitro* regardless of its phosphorylation state, but only the phosphorylated form of the protein can activate transcription (35). Light-modulated protein phosphorylation and subsequent regulation of gene expression have been demonstrated for certain phytochrome-regulated plant genes (13). Control by phosphorylation of chromatic adaptation in *F. diplosiphon* is suggested by the finding that *rcaC*, a gene whose function is necessary for response to red light, encodes a bacterial response regulator (7); activity of this family of proteins is typically regulated by phosphorylation (22, 32). Preliminary data suggest that phosphorylation is required for the *psbAII*-binding activity (20).

One G residue upstream of the transcription start site, within the promoter region (-18 in Fig. 5A and C), was also clearly protected *in vivo* under high light; we believe that this represents the binding of RNA polymerase to the *psbAII* promoter. This view is in agreement with the finding that DNase I footprinting shows RNA polymerase binding to the promoter region of the *cpeBA* operon in the cyanobacterium *Calothrix* sp. strain PCC 7601 (31). A few nucleotides around +1 that are neither A nor G were protected in Fig. 5A. This may be an artifact caused by random stopping of the DNA polymerase during the primer extension reaction (see reference 25). Analysis of the upper strand by primer extension (Fig. 5A) produced different patterns between the high- and low-light samples, even when the naked DNA was treated with DMS (compare lanes 2 and 5 in Fig. 5A). The bands that differed between the two patterns did not include those which were protected by *in vivo* DMS treatment. No differences were detected between the high- and low-light *in vitro*-treated samples for the other strand (Fig. 5B).

Our deletion mutant studies showed that although the 5-bp deletion from the untranslated leader region of *psbAII* did not alter the *in vitro* binding characteristics, the *in vivo* high-light induction of this mutant *psbAII* was only half that of the wild type (Fig. 7). It is possible that the correct phase of the binding protein on the DNA helix is required for fully induced *psbAII* expression under high light and that the phase change caused by the 5-bp deletion resulted in impaired high-light induction. The delta 5 mutation may have some effect on *in vitro* binding

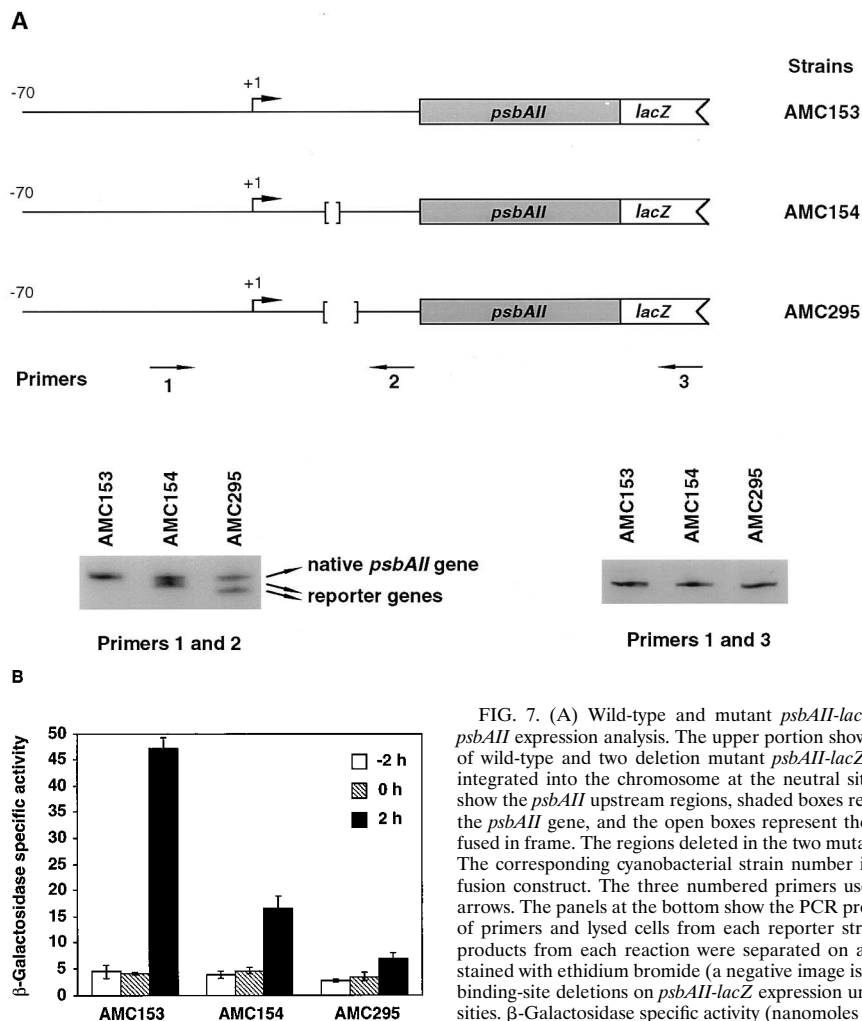


FIG. 7. (A) Wild-type and mutant *psbAII-lacZ* reporter fusions used for *psbAII* expression analysis. The upper portion shows a schematic representation of wild-type and two deletion mutant *psbAII-lacZ* fusion constructs that were integrated into the chromosome at the neutral site. The solid horizontal lines show the *psbAII* upstream regions, shaded boxes represent the first 20 codons of the *psbAII* gene, and the open boxes represent the truncated *E. coli lacZ* gene fused in frame. The regions deleted in the two mutants are indicated by brackets. The corresponding cyanobacterial strain number is shown at the right of each fusion construct. The three numbered primers used for PCR are indicated by arrows. The panels at the bottom show the PCR products generated by two pairs of primers and lysed cells from each reporter strain as a template. The PCR products from each reaction were separated on a 5% polyacrylamide gel and stained with ethidium bromide (a negative image is shown). (B) Effect of *psbAII*-binding-site deletions on *psbAII-lacZ* expression under low and high light intensities. β -Galactosidase specific activity (nanomoles of *o*-nitrophenyl- β -D-galactopyranoside per minute per milligram of protein) was determined for each strain. Bars show β -galactosidase levels for each strain 2 h before (white bars), at the time of (hatched bars), and 2 h after (black bars) the light was shifted from low to high intensity. Error bars represent standard deviation from the mean derived from three independent light shift experiments.

as well, since an altered binding pattern was observed in a subset of experiments for this probe fragment but never for the wild-type fragment. The deletion of a 12-bp sequence from the *psbAII* regulatory region in delta 12 eliminated one of the two DNA-protein complexes and significantly reduced the high-light induction *in vivo*, strongly indicating that the deleted sequences are necessary for light-responsive regulation. Combination with the *conII* promoter in a transcriptional *lacZ* fusion construct showed that enhancer activity was also affected by the mutation. More detailed mutational analyses within the region should provide conclusive answers as to whether the enhancer activity and high-light induction can be separated. A complete view of the signal transduction pathway still awaits identification of the *trans*-acting factors and other proteins, but our current data clearly demonstrate that the specific binding of protein(s) to the untranslated leader region of *psbAII* is important for its induced expression under high light.

ACKNOWLEDGMENTS

We thank Bob Haselkorn for providing antiserum directed against the core and sigma subunits of the *Anabaena* sp. strain PCC 7942 RNA polymerase.

This research was supported by Public Health Service grant R01 GM 37040 from the National Institutes of Health and National Science Foundation grant MCB-8958089. The dye sublimation printer and Fujix phosphorimaging system were purchased with funds from National Science Foundation Instrumentation and Instrument Development grant BIR-9217251.

REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
2. Bustos, S. A., and S. S. Golden. 1991. Expression of the *psbDII* gene in *Synechococcus* sp. strain PCC 7942 requires sequences downstream of the transcription start site. *J. Bacteriol.* **173**:7525-7533.
3. Bustos, S. A., M. R. Schaefer, and S. S. Golden. 1990. Different and rapid responses of four cyanobacterial *psbA* transcripts to changes in light intensity. *J. Bacteriol.* **172**:1998-2004.
4. Casadaban, M. J., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **138**:179-207.

5. Casey, E. S., and A. Grossman. 1994. In vivo and in vitro characterization of the light-regulated *cpcB2A2* promoter of *Fremyella diplosiphon*. *J. Bacteriol.* **176**:6362–6374.
6. Chastain, C. J., J. S. Brusca, T. S. Ramasubramanian, T.-F. Wei, and J. W. Golden. 1990. A sequence-specific DNA-binding factor (VF1) from *Anabaena* sp. strain PCC 7120 vegetative cells binds to three adjacent sites in the *xisA* upstream region. *J. Bacteriol.* **172**:5044–5051.
7. Chiang, G. G., M. R. Schaefer, and A. R. Grossman. 1992. Complementation of a red-light-indifferent cyanobacterial mutant. *Proc. Natl. Acad. Sci. USA* **89**:9415–9419.
8. Fujiwara, S., N. A. Zielinski, and A. M. Chakrabarty. 1993. Enhancer-like activity of AlgR1-binding site in alginate gene activation: positional, orientational, and sequence specificity. *J. Bacteriol.* **175**:5452–5459.
9. Gober, J. W., and L. Shapiro. 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3' enhancer and IHF binding elements. *Mol. Biol. Cell* **3**:913–926.
10. Golden, S. S. 1994. Light-responsive gene expression and the biochemistry of the photosystem II reaction center, p. 693–714. *In* D. A. Bryant (ed.), *The molecular biology of cyanobacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. Golden, S. S., J. Brusslan, and R. Haselkorn. 1987. Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol.* **153**:215–231.
12. Grossman, A. R., M. R. Schaefer, G. G. Chiang, and J. L. Collier. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiol. Rev.* **57**:725–749.
13. Harter, K., S. Kircher, H. Frohnmeyer, M. Krenz, and F. Nagy. 1994. Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. *Plant Cell* **6**:545–559.
14. Kim, J., C. Zwieh, C. Wu, and S. Adhya. 1989. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene* **85**:15–23.
15. Kulkarni, R. D., and S. S. Golden. 1994. Adaptation to high light intensity in *Synechococcus* sp. strain PCC 7942: regulation of three *psbA* genes and two forms of the D1 protein. *J. Bacteriol.* **176**:959–965.
16. Kulkarni, R. D., M. R. Schaefer, and S. S. Golden. 1992. Transcriptional and posttranscriptional components of *psbA* response to high light intensity in *Synechococcus* sp. strain PCC 7942. *J. Bacteriol.* **174**:3775–3781.
17. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
18. Kuwabara, M. D., and D. S. Sigman. 1987. Footprinting DNA-protein complexes in situ following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia* RNA polymerase-*lac* promoter complexes. *Biochemistry* **26**:7234–7238.
19. Li, R., and S. S. Golden. 1993. Enhancer activity of light-responsive regulatory elements in the untranslated leader regions of cyanobacterial *psbA* genes. *Proc. Natl. Acad. Sci. USA* **90**:11678–11682.
20. Li, R., and S. S. Golden. Unpublished data.
21. Mueller, U. W. 1991. Masters thesis. Texas A&M University, College Station.
- 21a. Nalty, M., and S. S. Golden. Unpublished data.
22. Parkinson, J. S. 1993. Signal transduction schemes of bacteria. *Cell* **73**:857–871.
23. Ramasubramanian, T. S., T.-F. Wei, and J. W. Golden. 1994. Two *Anabaena* sp. strain PCC 7120 DNA-binding factors interact with vegetative cell- and heterocyst-specific genes. *J. Bacteriol.* **176**:1214–1223.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. Sasse-Dwight, S., and J. D. Gralla. 1991. Footprinting protein-DNA complexes *in vivo*. *Methods Enzymol.* **208**:146–168.
26. Schaefer, M. R., and S. S. Golden. 1989. Differential expression of members of a cyanobacterial *psbA* gene family in response to light. *J. Bacteriol.* **171**:3973–3981.
27. Schmidt-Goff, C. M., and N. A. Federspiel. 1993. In vivo and in vitro footprinting of a light-regulated promoter in the cyanobacterium *Fremyella diplosiphon*. *J. Bacteriol.* **175**:1806–1813.
28. Schulze-Lefert, P., J. L. Dangel, M. Becker-Andre, K. Hahlbrock, and W. Schulz. 1989. Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J.* **8**:651–656.
29. Shapira, S. K., J. C. Chou, F. V. Richard, and M. J. Casadaban. 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of β -galactosidase. *Gene* **25**:71–82.
30. Sobczyk, A., A. Bely, N. Tandeau de Marsac, and J. Houmard. 1994. A phosphorylated DNA-binding protein is specific for the red-light signal during complementary chromatic adaptation. *Mol. Microbiol.* **13**:875–885.
31. Sobczyk, A., G. Schyns, N. Tandeau de Marsac, and J. Houmard. 1993. Transduction of the light signal during complementary chromatic adaptation in the cyanobacterium *Calothrix* sp. PCC 7601: DNA-binding proteins and modulation by phosphorylation. *EMBO J.* **12**:997–1004.
32. Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53**:450–490.
33. Tandeau de Marsac, N. 1983. Phycobilisomes and complementary chromatic adaptation in cyanobacteria. *Bull. Inst. Pasteur* **81**:201–254.
34. Tsinoremas, N. F., M. R. Schaefer, and S. S. Golden. 1994. Blue and red light reversibly control *psbA* expression in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J. Biol. Chem.* **269**:16143–16147.
35. Weiss, D. S., K. E. Klose, T. R. Hoover, A. K. North, C. S. Porter, A. B. Wedel, and S. Kustu. 1991. The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. *Cell* **67**:155–167.