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# Expression of the *psbDII* Gene in *Synechococcus* sp. Strain PCC 7942 Requires Sequences Downstream of the Transcription Start Site

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**The *psbDI* and *psbDII* genes in *Synechococcus* sp. strain PCC 7942 encode the D2 polypeptide, an essential component of the photosystem II reaction center. Previous studies have demonstrated that transcripts from *psbDII*, but not *psbDI*, increase in response to high light intensity. Soluble proteins from *Synechococcus* cells shifted to high light were found to have affinity for DNA sequences upstream from the *psbDII* coding region. DNA mobility-shift and copper-phenanthroline footprinting assays of a 258-bp fragment revealed three distinct DNA-protein complexes that mapped to the untranslated leader region between +11 and +84. Deletion of the upstream flanking region to −42 had no effect on the expression of a *psbDII-lacZ* reporter gene or its induction by light, whereas a promoterless construct supported only minimal background levels of β-galactosidase. A 4-bp deletion within the first protected region of the footprint decreased the β-galactosidase activity to approximately 2% of that of the undeleted control, but gene expression remained responsive to light. Deletion of the three protected regions completely abolished both gene expression and light induction. These results suggest that the *psbDII* gene requires elements within the untranslated leader region for efficient gene expression, one of which may be involved in regulation by light.**

Cyanobacteria make up a large and diverse group of photosynthetic bacteria. They share with plants the ability to use two photosynthetic reaction centers linked in series, termed photosystems I and II, to reduce photooxidized chlorophyll with electrons obtained from water, releasing molecular oxygen as a by-product. Components of the photosynthetic apparatus of cyanobacteria are highly conserved with their counterparts in the chloroplasts of higher plants (4). The reaction center core of photosystem II contains a dimer of two structurally similar proteins, D1 and D2 (48), which are encoded by the *psbA* and *psbD* genes, respectively (4). D1 and D2 house the photoreactive chlorophyll, primary acceptor, and other cofactors involved in electron transport through photosystem II (25, 33, 48). Both *psbA* and *psbD* are unique genes in the chloroplast genomes of most plants (19, 36, 41, 53) but are present in more than one copy in cyanobacterial genomes (9, 15, 18).

*Synechococcus* sp. strain PCC 7942 (hereafter referred to as *Synechococcus* sp.) contains three *psbA* genes and two *psbD* genes (15, 18). The *psbA* genes encode two distinct forms of the D1 protein (15). In contrast, the *psbD* genes encode identical D2 polypeptides (18); the two nucleotide differences in the coding sequences occur at redundant third-base positions. The *psbDI* gene overlaps and is cotranscribed with a unique photosystem II gene, *psbC*, which encodes a chlorophyll *a*-binding protein termed CP43 (4, 18). The *psbDII* gene is not adjacent to *psbDI-psbC* and is expressed as a monocistronic message. The expression of the *psbD* genes has been investigated by analysis of *psbDI-psbC* and *psbDII* transcripts and of β-galactosidase activity produced by *psbD-lacZ* translational fusions (6). The *psbDI* gene is expressed constitutively, whereas the *psbDII* gene is induced up to fivefold by a shift from moderate to high light intensity (6).

Extensive genetic and molecular analyses of prokaryotic regulatory systems have identified several classes of positive and negative control elements that influence transcription via interaction of *trans*-acting factors with *cis*-acting elements. Positive factors include the $NR_I$, AraC, CAP, MalT, and OxyR proteins in *Escherichia coli* (35, 39, 45); negative factors include repressor proteins for the *gal*, *ara*, and *lac* operons (38). In both types of control, the regulatory factor is a DNA-binding protein with high affinity for specific sites located near the promoter (38).

Here we show that DNA-binding proteins from *Synechococcus* cells shifted to high light bind specifically to three sites within the untranslated leader region of the *psbDII* gene. In addition, we establish that a 63-bp sequence located between +20 and +82 bp from the *psbDII* transcription initiation site is necessary to maintain gene expression. Our experiments indicate that the *psbDII* promoter requires the interaction of DNA-binding proteins with *cis* regulatory sequences to mediate efficient gene expression.

## MATERIALS AND METHODS

**Strains and plasmids.** Strains and plasmids used in this work are described in Table 1. Numerical designations of nucleotides used in the construction of plasmids were assigned in relation to the transcription start site (+1). Wild-type *Synechococcus* sp. strain PCC 7942 (previously referred to as *Anacystis nidulans* R2, Pasteur Culture Collection no. 7942) and mutant strains were grown in liquid BG-11 medium (1) with the following modifications: no $Na_2SiO_3 \cdot 9H_2O$ was added, and 0.012 g of $FeNH_4$ citrate per liter was used instead of 0.006 g of citric acid per liter and 0.006 g of cupric citrate per liter. Modified BG-11 was solidified with agar as previously described by Golden et al. (16).

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| *Synechococcus* strains | | |
| PCC 7942 | Wild type (also called *Anacystis nidulans* R2) | Laboratory collection |
| AMC099 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in *Synechococcus* chromosome; upstream flanking DNA of reporter gene extends to −96, with a 4-bp deletion in the untranslated leader region | This study |
| AMC125 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in the *Synechococcus* chromosome; reporter gene is that of AMC099 but has a 60-bp deletion within the untranslated leader region of *psbDII* | This study |
| AMC126 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in the *Synechococcus* chromosome; reporter gene is that of AMC099 but has a 23-bp deletion within the untranslated leader region of *psbDII* | This study |
| AMC127 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in the *Synechococcus* chromosome; upstream flanking DNA of reporter gene extends to −196 of *psbDII* | This study |
| AMC128 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in the *Synechococcus* chromosome; upstream flanking DNA of reporter gene extends to −42 bp of *psbDII* | This study |
| AMC129 | *psbDII-lacZ* reporter gene fusion with a deleted promoter integrated at a neutral site in the *Synechococcus* chromosome; upstream flanking DNA of reporter gene extends to +25 of the *psbDII* untranslated leader region | This study |
| AMC132 | *psbDII-lacZ* reporter gene fusion integrated at a neutral site in the *Synechococcus* chromosome; upstream flanking DNA of reporter gene extends to −406 of *psbDII* | 6 |
| Plasmids | | |
| pUC18 | Cloning vector, encodes ampicillin resistance | 52 |
| pBluescript KS+ | Cloning vector, encodes ampicillin resistance | Stratagene |
| pBGS19 | Derivative of pUC19, encodes kanamycin resistance | 43 |
| pBR328 | Cloning vector, encodes ampicillin, tetracycline, and chloramphenicol resistance | 42 |
| pAM697 | pBGS19 with a 434-bp *Bam*HI-*Hin*cII fragment that extends from −242 to +192 of the *psbDI* gene; insert used in mobility-shift assays | This study |
| pAM736 | *Synechococcus* recombinational cloning vector; pBR328 with 2.8-kb *Bam*HI *Synechococcus* fragment that carries a modified spectinomycin resistance (Ω) cassette at a unique *Xho*I site; *psbDII* reporter gene constructs were cloned at a *Sma*I site located at the 3′ end of the Ω cassette | 6 |
| pAM737 | pBluescript KS+ with 258-bp *Rsa*I-*Taq*I fragment that extends from −96 to +162 of the *psbDII* gene; insert used as probe in mobility-shift assays | This study |
| pAM792 | pBluescript KS+ with a *psbDII-lacZ* gene fusion; upstream flanking DNA of reporter gene extends to −196 of *psbDII* | This study |
| pAM854 | Similar to pAM736 but with the Ω cassette in the opposite orientation | 6 |
| pAM867 | pUC18 with a 116-bp *Rsa*I-*Alu*I fragment from the *psbDII* upstream region; used to construct the deletions within the untranslated region of the *psbDII-lacZ* reporter gene | This study |
| pAM869 | pBluescript KS+ with a *psbDII-lacZ* gene fusion; upstream flanking DNA of reporter gene extends to −96 bp of *psbDII* with a 4-bp deletion in the untranslated leader region | This study |
| pAM870 | Similar to pAM869 but has a 60-bp deletion within the untranslated leader region | This study |
| pAM871 | Similar to pAM869 but has a 23-bp deletion within the untranslated leader region | This study |
| pAM872 | pAM854 with a *psbDII-lacZ* gene fusion; upstream flanking DNA of reporter gene extends to −96 bp of *psbDII* with a 4-bp deletion in the untranslated leader region; recombinational substrate to produce AMC099 | This study |
| pAM873 | Similar to pAM872 but has a 60-bp deletion within the untranslated leader region; recombinational substrate to produce AMC125 | This study |
| pAM874 | Similar to pAM872 but has a 23-bp deletion within the untranslated leader region; recombinational substrate to produce AMC126 | This study |
| pAM899 | pAM736 with *psbDII-lacZ* gene fusion; upstream flanking DNA of reporter gene extends to −196 bp of *psbDII*; recombinational substrate to produce AMC127 | This study |
| pAM900 | pAM736 with *psbDII-lacZ* gene fusion; upstream flanking DNA of reporter gene extends to −42 bp of *psbDII*; recombinational substrate to produce AMC128 | This study |
| pAM902 | pAM736 with *psbDII-lacZ* gene fusion having the *psbDII* promoter completely deleted; upstream flanking DNA of reporter gene extends to +25 bp; recombinational substrate to produce AMC129 | This study |

A detailed procedure for transformation and gene inactivation of *Synechococcus* sp. has been published (16). Cyanobacterial transformants containing a modified Ω cassette (34) were selected on a combination of 40-μg/ml spectinomycin and 5-μg/ml streptomycin to avoid spontaneous spectinomycin-resistant mutants. Thereafter, transformed strains were maintained on spectinomycin alone. *E. coli* DH5α (*mcrA mcrB*) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), used as the host for all plasmids, was propagated on Luria-Bertani broth (LB) (23) and Terrific Broth (47) media in the presence of antibiotics at standard concentrations (23).

***Synechococcus* culture conditions.** For controlled light studies, wild-type *Synechococcus* sp. and mutant strains were cultured in a turbidostat (6) or in Pyrex tubes (2-cm diameter, 17 cm long) submerged in an aquarium containing

distilled water at a constant temperature of 30°C. Cultures were bubbled with a constant source of 1% $CO_2$ in air. Light was provided by six 34-in. (86-cm) 20-W fluorescent tubes placed 15 cm from the aquarium. An immersible probe (Biospherical Instruments, San Diego, Calif.) was used to measure the photosynthetic photon flux density (measured in microeinsteins per square meter per second) reaching the *Synechococcus* cell cultures. For light-shift experiments involving β-galactosidase assays, reporter strains were first grown in 250-ml flasks in a chamber at 100 microeinsteins · $m^{-2}$ · $s^{-1}$ for several days. Samples were transferred to Pyrex tubes, and the $A_{750}$ was standardized to 0.2 by the addition of fresh medium. Tubes were placed in the aquarium, and cells were cultured at 125 microeinsteins · $m^{-2}$ · $s^{-1}$ for 24 h, or until the $A_{750}$ was between 0.4 and 0.6. At time zero, a sample was removed, the remaining culture was shifted to 720 microeinsteins · $m^{-2}$ · $s^{-1}$, and additional samples were taken 2 h after the shift to high light. Samples were harvested by centrifugation and frozen immediately at −90°C. Cells were lysed by the method of Schaefer and Golden (40), and the β-galactosidase activity in the aqueous phase was determined by colorimetric assay with *o*-nitrophenyl-β-D-galactopyranoside by the method of Miller (27) and expressed as specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein).

**DNA methods.** Most restriction and modifying enzymes were purchased from Bethesda Research Laboratories or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as directed by the manufacturers. The total DNA small-scale procedure used to screen cyanobacterial transformants was done as described previously (16) and modified by Bustos et al. (7).

Southern blot analysis was performed by transferring digested DNA from agarose gels (0.7% [wt/vol] in 0.5× Tris-borate-EDTA (TBE) buffer [23]) to charged nylon membranes (Magnagraph; Micron Separation Inc., Westborough, Mass.), using a vacuum blotter (Vacublot; American Bionetics Inc., Hayward, Calif.) and a solution of 10× SSPE (23) (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) as a transfer medium. Hybridization was performed by the method of Golden et al. (17). Unhybridized probes were removed from the filters by washing in 0.5× SSPE–0.2% sodium dodecyl sulfate at 65°C. Radioactive DNA probes were produced from gel-purified DNA fragments (3) by random primer labeling with a commercial kit (Bethesda Research Laboratories) and [α-$^{32}$P]dCTP.

**Deletions upstream of the *psbDII* coding region.** The plasmid pAM792 (Table 1), containing the *psbDII-lacZ* gene fusion and upstream flanking DNA to nucleotide −196, was digested with exonuclease III (Erase-a-base kit; Promega, Madison, Wis.) to generate upstream deletion clones. Specific protein-binding sites were deleted by fusing the desired exonuclease III products to pAM867 (Table 1), which had been digested with *Kpn*I. This plasmid contains a 116-bp *Rsa*I-*Alu*I fragment from the upstream region of the *psbDII* gene that includes the promoter and part of the untranslated leader region. Six, 25, and 63 nucleotides of the native sequences were removed and 2, 2, and 3 bp were added, respectively, within the upstream leader of the *psbDII-lacZ* gene fusion. The lengths of these deletions will be referred to as 4, 23, and 60 bp. The deletion clones are contained in plasmids pAM869, pAM871, and pAM870, respectively. The junction sequences were verified by dideoxy chain termination sequencing reactions using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Double-stranded sequence templates were prepared as described by Morden and Golden (29).

**Preparation of soluble protein extracts from *Synechococcus* sp.** Soluble protein extracts were enriched for DNA-binding proteins by the method of Chastain et al. (8), with the following modifications. *Synechococcus* cells were cultured in a turbidostat (6) under 125 microeinsteins of white light · $m^{-2}$ · $s^{-1}$ and at an $A_{750}$ of 0.65 to 0.8 for several days. The fluorescent light bulbs were then moved to the closest position to the culture vessel, which provided a photosynthetic photon flux density of 320 microeinsteins · $m^{-2}$ · $s^{-1}$. This range of light intensity has been shown to induce expression of the *psbDII* gene (6). After 30 min at the higher light intensity, cells from a 750-ml sample were harvested by centrifugation at 4,000 × *g* for 10 min. All purification steps were done at 0 to 4°C. The pelleted cells were resuspended in 10 ml of extraction buffer (8) and homogenized for 3 min in a homogenizing mill (Braun) chilled with liquid $CO_2$. The cell lysate was centrifuged at 31,000 × *g* for 10 min, and the supernatant fraction was centrifuged at 142,000 × *g* for 60 min. The supernatant fraction from the latter centrifugation was bound to a heparin-Sepharose column and eluted with a 44-ml linear gradient of 0.1 to 1.0 M ammonium sulfate in buffer A (8). Fractions (2 ml) were collected and dialyzed against 2.5 liters of 50 mM Tris-HCl (pH 7.5)–0.1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–10% glycerol. Samples from each column fraction (5 μl) were assayed for DNA-binding activity in DNA mobility-shift assays.

**DNA mobility-shift assays.** DNA mobility-shift assays were performed as described by Ausubel et al. (3). Samples containing 0.2 to 0.5 ng of labeled DNA fragment were incubated with 5 μl of a heparin-Sepharose column fraction in binding buffer (8) containing 0.5 μg of poly(dI-dC) in a final volume of 20 μl. After 30 min at room temperature, samples were loaded onto a 5% polyacrylamide gel (30:1 acrylamide-bisacrylamide, 50 mM Tris chloride, 380 mM glycine, 2.5% glycerol). Electrophoresis was performed at room temperature for 20 min at 200 V in a Tris-glycine running buffer (3) using a Mini-protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). Gels were dried and exposed to X-ray film.

The DNA fragments used in the mobility-shift assays were obtained by digestion of appropriate plasmids with restriction enzymes that leave overhanging 5′ ends. The fragments were 3′ labeled by filling in unpaired bases with [α-$^{32}$P]dCTP and other deoxynucleoside triphosphates, using the Klenow fragment of DNA polymerase I. Competition experiments were performed by adding unlabeled competitor DNA fragments to mobility-shift assay reactions at the same time as the labeled DNA and incubating at room temperature for 30 min.

**Cu-Op DNA footprinting.** The plasmid pAM737 was digested with *Xba*I or *Hin*dIII and radiolabeled as described above; the *Rsa*I-*Taq*I fragment was then released by digesting with an enzyme at the other end of the multiple cloning site. Each fragment was excised and electroeluted from a 5% acrylamide gel (3), and approximately 30,000 cpm was incubated with 42 μl of a heparin-Sepharose fraction that showed peak binding activity in mobility-shift assays. Binding reactions (50 μl) and electrophoresis were performed as described above. Copper-phenanthroline (Cu-Op) cleavage reactions were performed in the acrylamide matrix by the method of Kuwabara and Sigman (22). The reaction mixture was incubated at room temperature for 15 min, and then the reaction was quenched by the addition of 20 ml of 28 mM

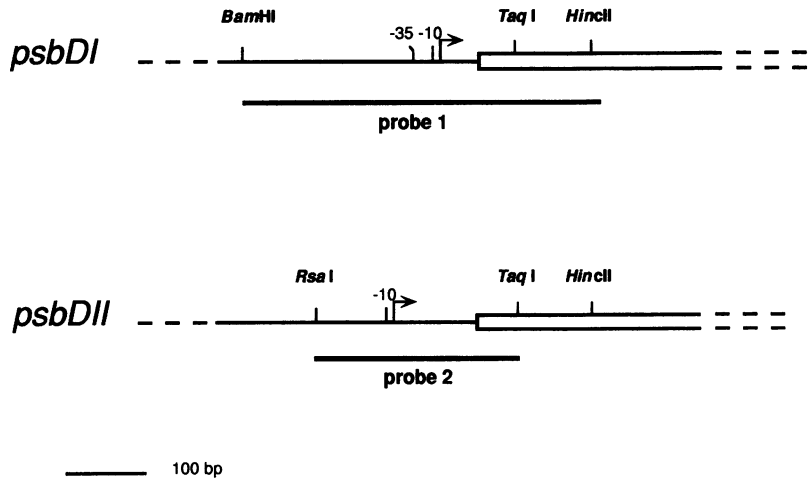


FIG. 1. Schematic representation of the *psbD* upstream regions. Horizontal lines represent the regions of the chromosome that contain the sequences upstream of each of the *psbD* genes. Restriction enzyme cleavage sites used to excise probes 1 and 2 are shown. A box open at the right end depicts each open reading frame. Sequences characteristic of *E. coli* promoter elements are indicated by −35 and −10, and the transcription start sites are shown by rightward arrows. The *psbDII* gene lacks a distinguishable −35 element. A dark bar under each gene represents the restriction fragment used in mobility-shift assays and footprinting analysis.

2,9-dimethyl-Cu-Op and allowed to stand for 2 min. The gels were rinsed with distilled water and exposed to X-ray film for 3 h. Bands of interest were excised from the gel and electroeluted overnight at room temperature in 0.5× TBE (3). Eluted DNA was extracted with phenol, with equal parts of phenol and chloroform-isoamyl alcohol (24:1), and again with chloroform-isoamyl alcohol. DNA was then ethanol precipitated and dried. A sample of the eluted labeled DNA (approximately 5,000 to 10,000 cpm) was resuspended in 6 μl of a solution containing 80% (vol/vol) formamide, 10 mM NaOH, 1.0 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol and loaded on a 6% sequencing gel. The protected bases were identified from a sequencing ladder generated by chemical cleavage reactions of the same labeled DNA fragment (26).

## RESULTS

**Partial purification of DNA-binding proteins.** We have previously shown that the *Synechococcus psbDII* gene responds to changes in light intensity (6). Experiments using *psbD-lacZ* reporter strains as well as Northern (RNA) blot analysis of wild-type transcripts indicate that a regulatory component of *psbDII* expression is at the transcriptional level. These results prompted experiments to detect possible interactions of *trans*-acting proteins with *cis*-acting elements and to determine the roles of these factors in the regulation of *psbD* gene expression.

Figure 1 shows the upstream regions of the *psbD* genes, the transcription and translation start sites, and the DNA probes used in mobility-shift assays. *Synechococcus* protein extracts were enriched for DNA-binding proteins by heparin-Sepharose column chromatography (5). Samples eluted from a linear 0.1 to 1.0 M ammonium sulfate gradient were examined for binding to DNA by mobility-shift assays with fragments containing sequences upstream from the *psbDI* and *psbDII* coding regions (Fig. 1, probes 1 and 2). Protein-DNA complexes (designated C1′, C1, C2, and C3 in increasing relative mass) appeared as distinct bands on the autoradiogram only with probe 2, which contains upstream regions of the *psbDII* gene (Fig. 2B). The presence and/or intensity of C1′ (which is also visible in Fig. 3 and 6) varied with different protein extract preparations; thus, we chose not to include it in footprinting reactions that follow.

The observed complexes were abolished by proteinase K or heat treatment (80°C, 10 min) of the selected column fractions but not by treatment with RNase A (data not shown). Minor bands, which were attributed to partial denaturation or secondary structure of the labeled DNA, sometimes were observed associated with the free probe (Fig. 2A, lane N). Other DNA fragments capable of forming complexes with the column fractions shared the region that extends from −96 to +162 relative to the transcription start site of the *psbDII* gene (data not shown). These experiments suggested that probe 2 contained all the binding sites and

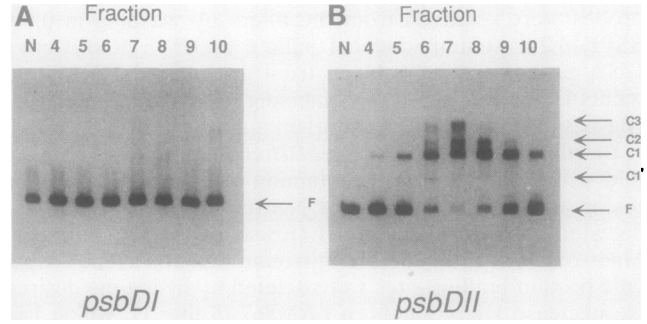


FIG. 2. DNA mobility-shift assays of *psbDI* and *psbDII* upstream fragments with a cell lysate enriched for DNA-binding proteins. Soluble protein extracts from *Synechococcus* cells adapted to 125 microeinsteins · $m^{-2}$ · $s^{-1}$ and shifted to 320 microeinsteins · $m^{-2}$ · $s^{-1}$ for 30 min were enriched for DNA-binding proteins by heparin-Sepharose chromatography and incubated with DNA fragments from the upstream regions of the *psbDI* (A) or *psbDII* (B) gene. Column fractions were tested for binding with a 434-bp *psbDI* DNA probe (Fig. 1, probe 1) and a 258-bp *psbDII* DNA probe (Fig. 1, probe 2). Column fractions are marked above each lane. Lanes marked N contain no extract and show the migration of the free probe, F. The migration of DNA-protein complexes is indicated as C1′, C1, C2, and C3.

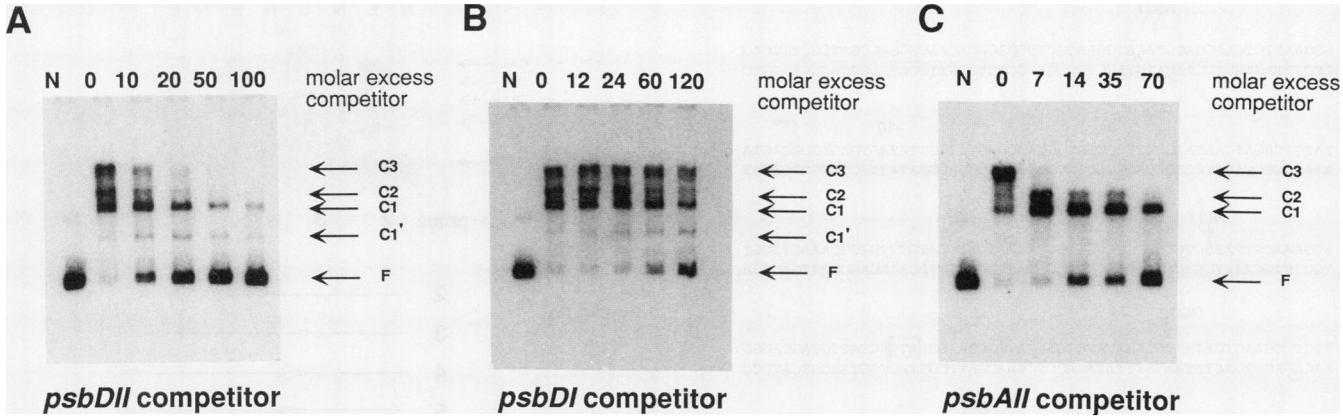


FIG. 3. Competition of regulatory regions from other genes for proteins that bind to upstream sequences of the *psbDII* gene. DNA mobility-shift assays are shown for probe 2 (Fig. 1) incubated with heparin-Sepharose fraction 7 (Fig. 2B, lane 7) and competed with unlabeled DNA. Panels show competition of probe 2 with a probe 2 fragment (A), with a probe 1 fragment (B), and with a 210-bp *psbAII* gene fragment (C). Lanes N contain no extract. All other lanes contain the indicated molar excess of unlabeled competitor fragment; each panel is marked with the source of competing DNA. Arrows mark the migration of the free probe, F, and DNA-protein complexes C1′, C1, C2, and C3.

could be used for mobility-shift assays and footprinting experiments to localize the sites of protein binding.

Peak complex formation with probe 2 in mobility-shift assays was observed with proteins that eluted between 0.3 and 0.35 M ammonium sulfate (Fig. 2B, lanes 6 and 7). The 434-bp labeled *Bam*HI-*Hin*cII DNA fragment that contains upstream regions of the *psbDI* gene (Fig. 1, probe 1) failed to form complexes with any of the column fractions under the same experimental conditions (Fig. 2A). These results suggest that DNA-binding proteins are involved in the mechanism of light-responsive expression of the *psbDII* gene.

**DNA binding competition experiments.** To assess the specificity of binding, we used unlabeled restriction fragments from the upstream regions of *psbDI*, *psbDII*, and *psbAII* to compete with probe 2 for DNA-binding proteins. A 20-fold molar excess of unlabeled probe 2 fragment successfully competed for C3 and C2 formation, but a 100-fold excess was necessary to compete for C1 formation (Fig. 3A). An unlabeled probe 1 fragment (Fig. 1, probe 1) failed to compete with probe 2 for *psbDII*-binding proteins even at a 120-fold excess (Fig. 3B). A 210-bp DNA fragment that contains the upstream region and part of the open reading frame of the *psbAII* gene competed with probe 2 for the formation of the C3 complex at only sevenfold excess. A 35-fold excess competed for formation of C3 and C2; C1 remained unaltered even at a 70-fold excess of the *psbAII* competitor (Fig. 3C). These results show that proteins binding to *psbDII* DNA do not interact efficiently with the *psbDI* DNA fragment but bind strongly to the upstream region of the *psbAII* gene. The *psbAII* gene encodes the D1 protein, the other member of the photosystem II reaction center, and is also induced by a shift to high light intensity (7).

**Localization of DNA-protein complexes.** The Cu-Op technique (22) was used to define the sites of protein-DNA interaction more precisely within the 258-bp probe 2 fragment. Each strand of the cloned DNA fragment was radiolabeled at the adjacent *Xba*I or *Bam*HI site and incubated with fraction 7 of the *Synechococcus* extract for a mobility-shift assay. Bands corresponding to the C1, C2, and C3 complexes and unbound probe were treated with Cu-Op directly in the gel matrix. DNA purified from the combined complex and the unbound probe was analyzed on urea-polyacrylamide gels together with Maxam and Gilbert (26) sequencing ladders prepared from the same fragments. The upper and lower strands each showed three regions of protection from and two regions of hypersensitivity to Cu-Op cleavage (data not shown).

The three regions of DNA that exhibited various degrees of protection from Cu-Op digestion in the upper strand extended from nucleotides +11 to +25, +37 to +48, and +79 to +84, respectively (Fig. 4). In the lower strand, protection sites extended from nucleotides +11 to +23, +34 to +40, and +78 to +82, respectively. An additional region, which extended from +89 to +91, was identified only in the lower strand; these nucleotides may be part of the third binding site rather than a fourth site. Hypersensitivity to Cu-Op cleavage was observed at positions +13 and +21 in the upper and lower strands, respectively, roughly in the middle of the first protected region. All the protected regions were located between the transcription and translation start sites, and they do not overlap the putative ribosome-binding site (GGAGA) or the translation start codon (ATG) for the *psbDII* gene (Fig. 4).

**Deletion of protein-binding sites.** A series of clones was constructed in which one, two, or three protected regions were deleted. Because these regions were located in the upstream untranslated region, it was necessary to design internal deletions that did not remove the *psbDII* promoter. Exonuclease deletions were generated upstream of a *psbDII-lacZ* reporter gene and fused to a 116-bp *Rsa*I-*Alu*I fragment that extends from nucleotides −96 to +19 (Fig. 4). Restriction enzyme digestion at the *Alu*I site interrupts the first protected region, and this site was used as the leftward border of the internal deletions. The rightward borders and the small insertions that resulted from the cloning process are indicated by the downward arrows in Fig. 4. Thus, each deletion removed the nucleotides between the *Alu*I site and a given arrow and added the two or three bases shown next to the arrow.

The plasmids pAM869, pAM871, and pAM870 (Table 1) contain internal deletions of 4, 23, and 60 bp, respectively, beginning at nucleotide +20. Each deletion altered one, two, or all three protected DNA regions (Fig. 4). The various deleted *psbDII* DNA fragments were isolated as *Xba*I-*Sma*I restriction fragments from plasmids pAM869, pAM871, and

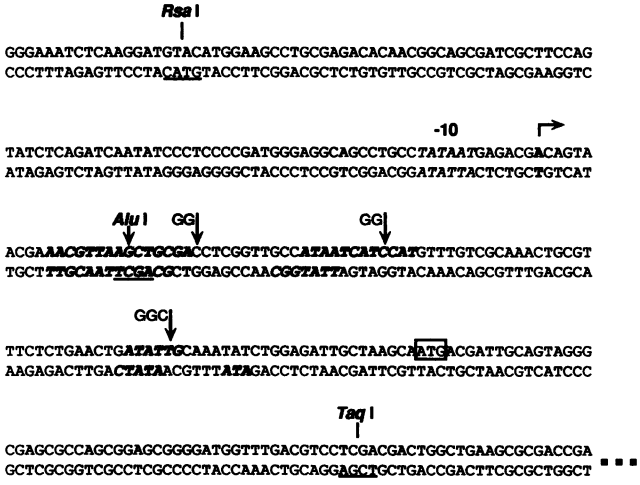


FIG. 4. Nucleotide sequence upstream of the *psbDII* gene in *Synechococcus* sp. Underlined sequences represent the restriction sites used to generate probe 2 and the binding-site deletion clones. Sequences characteristic of *E. coli* promoter elements are indicated by −10, and the transcriptional start site is shown by the rightward arrow and boldface nucleotide. The boxed letters ATG represent the translation start codon for the *psbDII* gene. Boldface italic letters represent DNA sequences protected from Cu-Op cleavage in the upper and lower strands. The *Alu*I site identifies the leftward border of the binding-site deletions; the rightward border of each deletion is shown by the downward arrows. The 2- or 3-bp insertions that resulted from the cloning process are shown to the left of each arrow. Thus, a given deletion clone is missing nucleotides between the arrow at the *Alu*I site and one other arrow, with insertion of the intervening bases.

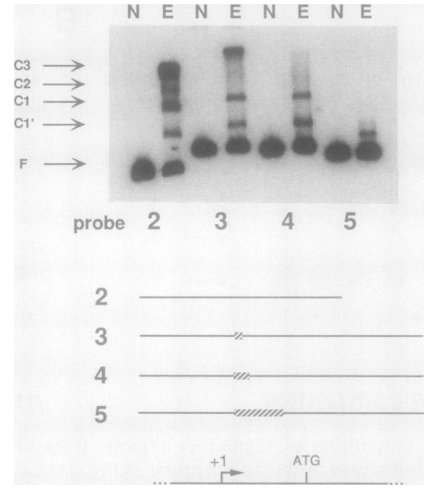


FIG. 5. DNA mobility-shift assays of the wild-type probe 2 fragment and DNA fragments deleted for one or more of the binding sites. All DNA probes were incubated with heparin-Sepharose column fraction 7 (Fig. 2B, lane 7). Lanes marked N contain no extract and show the migration of the free probe, F. Incubation with extract (lanes marked E) showed DNA-protein complexes indicated as C1′, C1, C2, and C3. Pairs of lanes labeled 2, 3, 4, and 5 used the probes shown at the bottom of the figure. Solid lines represent the region upstream from the *psbDII* gene. Hatched lines indicate the deleted regions. Sites for initiation of transcription (+1) and translation (ATG) are indicated. Probe 2, undeleted *psbDII* upstream fragment; probe 3, DNA fragment having 4 bp deleted from the first binding site; probe 4, DNA fragment having 23 bp deleted from the first and second binding sites; probe 5, DNA fragment containing a 60-bp deletion of the three binding sites. Precise endpoints of deleted sequences are shown in Fig. 4.

pAM870, radiolabeled at the *Xba*I site, and designated as probes 3, 4, and 5, respectively (Fig. 5). Each of these DNA fragments carried 93 additional base pairs from the *psbDII* open reading frame relative to probe 2. These additional sequences did not alter complex formation when tested with an undeleted probe (data not shown).

Figure 5 shows the results of mobility-shift assays of DNA fragments incubated with the same protein fraction used in the experiments shown in Fig. 2 and 3. The undeleted probe 2 formed the three distinct complexes observed previously: C1, C2, and C3 (Fig. 5). Probe 3, which has a 6-bp deletion and a 2-bp insertion within the first binding site, slightly modified the formation of one complex and was unaltered for formation of the other two (Fig. 5). Probe 4, which is deleted for the first and second binding sites, failed to form complexes C2 and C3 (Fig. 5) but retained C1. Probe 5, in which all three binding sites have been deleted, failed to form any of the three complexes (Fig. 5). Since the C1′ complex was formed even with probe 5, from which all known protected regions had been deleted, the C1′ binding site must be outside the 60-bp deleted fragment.

The results from these mobility-shift assays showed that the three *psbDII* binding sites are independent of each other, since removal of the promoter-proximal binding site did not significantly affect the formation of the two remaining complexes.

***psbDII* gene expression in vivo.** Each of the DNA fragments used in the mobility-shift assays was tested for its ability to drive expression of a *psbDII-lacZ* translational gene fusion to investigate the effect of the binding site deletions on expression of the *psbDII* gene in vivo (Fig. 6). A series of deletions with leftward endpoints corresponding to positions −406, −196, −42, and +25 (Fig. 6) were used to identify possible *cis*-regulatory elements located in this region, but not detectable by mobility-shift assays. Each deletion clone was recombined into the *Synechococcus* chromosome at a transcriptionally silent locus described elsewhere (6). The correct pattern of recombination was verified by Southern blot analysis (data not shown).

The effect of an increase in light intensity on expression of the deletion constructs was studied in a series of light-shift experiments. Strains were cultured at standard light intensity (100 microeinsteins · $m^{-2}$ · $s^{-1}$) for 24 h or until the culture reached a cell density that gave an $A_{750}$ reading between 0.4 and 0.6. At this time, a control sample was removed and the remaining culture was shifted to a higher light intensity (720 microeinsteins · $m^{-2}$ · $s^{-1}$) for 2 h, after which the cells were harvested for β-galactosidase assays.

Figure 7 depicts the β-galactosidase specific activities before and after the shift to high light. All strains deleted for sequences upstream of nucleotide −42 displayed similar patterns of expression and induction. Strains AMC132 and AMC128, having deletion endpoints at nucleotides −406 and −42, respectively, yielded β-galactosidase levels of approximately 140 U at normal light intensity that increased to 250 U 2 h after the transfer to high light intensity. Strain AMC127, having a deletion endpoint at nucleotide −196, had a lower basal level of expression but responded similarly to induction by light. Strain AMC129, containing a promoter deletion extending to nucleotide +25, produced only background levels of β-galactosidase (data not shown).

Strains missing one or more protein-binding sites dis-

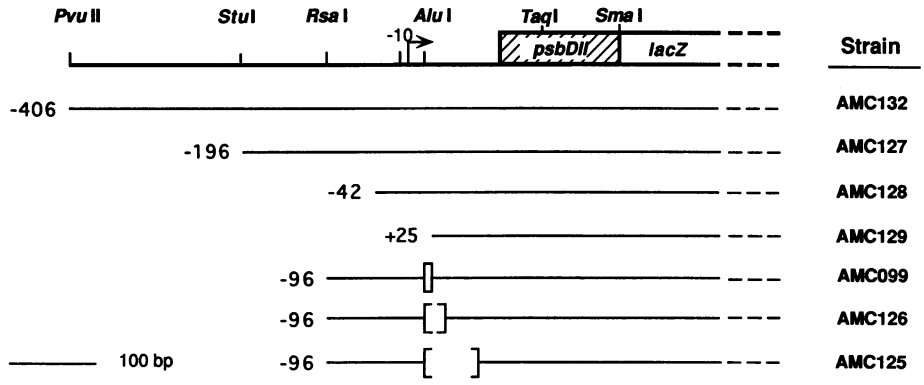


FIG. 6. Schematic representation of *psbDII-lacZ* reporter gene fusions used for the analysis of *psbDII* expression. Horizontal lines show the region upstream of *psbDII-lacZ* that is present in each construct. Nucleotide positions of leftward endpoints are indicated. Restriction enzyme cleavage sites used in the construction of the deletion clones are shown. The hatched box represents the first 48 codons of the *psbDII* gene, and the open box represents the truncated *E. coli lacZ* gene fused in frame. Internal deletions are indicated by brackets, and the corresponding *Synechococcus* strain numbers are shown at the right of the drawing.

played significantly lower β-galactosidase levels at both light intensities. The 4-bp deletion in strain AMC099 reduced the expression to 3 U above background at normal light intensity but allowed the activity to increase to 12 U 2 h after the shift to high light. The β-galactosidase levels in strain AMC126, which has two binding sites deleted, were somewhat higher than those of AMC099 both before and after the light shift. Deletion of all three binding sites in mutant strain AMC125 resulted in a complete loss of *psbDII-lacZ* gene expression, yielding β-galactosidase levels even lower than the background levels detected in the promoterless reporter strain, AMC129. This analysis indicates that the *psbDII* promoter requires the presence of *cis*-acting regulatory sequences located between the transcription and translation start sites for efficient expression of the *psbDII* gene. The promoter-distal binding site, or an additional protein-binding site outside of the 60-bp deleted region, may act with the promoter to confer the light response.

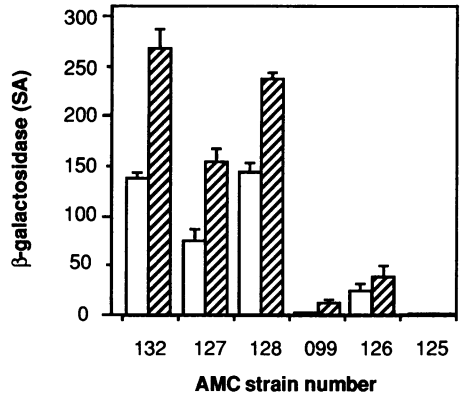


FIG. 7. Effect of *psbDII-lacZ* upstream deletions on β-galactosidase activity at moderate and high light intensities. β-Galactosidase specific activity (SA) (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein) was determined for each of the *psbDII-lacZ* gene fusions in light-shift experiments. Histogram bars show β-galactosidase levels for each mutant strain before (white bars) and 2 h after (hatched bars) the shift to high light intensity. β-Galactosidase levels were corrected by subtracting background levels from the *psbDII-lacZ* promoterless strain AMC129. The culture collection number for each reporter strain is indicated below. The values graphed are the means of triplicate experiments, with the standard deviations indicated.

## DISCUSSION

We combined DNA mobility-shift assays with in situ Cu-Op footprinting experiments to show that the region between +11 to +84 from the transcription start site of the *psbDII* gene binds proteins from a soluble cell extract of *Synechococcus* sp. The symmetrical heparin-Sepharose column elution profile (Fig. 2), the results from competition experiments with unlabeled probe 2 (Fig. 3A), and the shift to higher-molecular-weight complexes seen with increasing concentrations of DNA-binding proteins (data not shown) suggest that the same protein binds to more than one site in the DNA. However, unlabeled DNA from the upstream region of *psbAII* competed for formation of only the C2 and C3 complexes, even in the presence of a high molar excess of the competing fragment. Thus, at least two different proteins may be interacting with the *psbDII* protected regions, only one of which binds to *psbAII* DNA. Analysis of the binding site affinities and possible cooperative binding of the protein(s) that interact with the *psbDII* gene will require their purification and characterization.

A 210-bp *psbAII* DNA fragment forms two complexes when exposed to the same heparin-Sepharose column fraction that contains the *psbDII* DNA-binding protein(s) (31). These results and competition experiments suggest that the two genes bind the same, or a similar, protein(s). The *psbAII* gene, along with *psbAI* and *psbAIII*, encodes the D1 protein of the reaction center of photosystem II. Both the *psbAII* and *psbDII* genes are expressed at low levels when *Synechococcus* cells are grown at low light but increase their expression at high light intensities; this induction is at the transcriptional level (6, 7). These results suggest that the *psbAII* and *psbDII* genes are part of a regulon that coordinates expression of the D1 and D2 proteins in response to environmental signals. The DNA sequences of the upstream and untranslated leader regions of *psbDII* and *psbAII* do not show obvious identities except around the translation start site, where a minor footprint was detected on one strand of *psbDII* DNA (data not shown), and where a protein binds to *psbAII* DNA (31). The lack of a conserved DNA sequence

motif among binding sites has been reported previously for the *E. coli* regulatory protein OxyR (46).

Proteins binding to the probe 2 fragment provided weak protection from cleavage by the Cu-Op reagent. Although the protection was not dramatic, it was reproducible, and the affected segments of the two DNA strands aligned well. Correct assignment of the binding sites was supported by the results of small deletions in these regions that abolished formation of the predicted number of complexes in mobility-shift assays. The nucleotide sequences of the protected regions are A+T-rich, as are binding sites for the catabolite activator protein in the galactose and lactose operons of *E. coli* (21, 51). Sequences containing dyad symmetry or conserved motifs were not apparent. It may be that structural features of the A+T-rich DNA sequences are as important as a particular nucleotide sequence for protein binding. Such loose sequence binding requirements have been reported for yeast activator proteins (37). The location of proposed regulatory sequences between the transcription and translation start sites has also been observed for the binding of VF1 upstream of the *rbcL* gene of *Anabaena* sp. strain PCC 7120 (14). VF1 was first identified as a protein that binds upstream of the *Anabaena xisA* gene, but the *xisA* transcription start site has not been identified (8).

Similar patterns of DNA-protein complexes were obtained in mobility-shift assays with protein extracts from *Synechococcus* cells cultured at high or normal light intensities (data not shown), raising the possibility that the protected regions are not directly involved in light regulation. However, the complexes formed at the two light intensities may differ in ways not detectable by mobility-shift assays, such as by protein modification. Changes in light intensity could directly or indirectly modify a regulatory factor bound to a specific DNA sequence to switch the protein from an inactive to an active conformation at high light intensity. Other bacterial regulatory proteins bind close to or at the promoter of the genes that they regulate both in the presence and absence of inducer (13, 35, 44, 50). This design may be ideal for regulating responses that require immediate induction.

From the present study, we cannot conclude that the protein-binding sites participate directly in the light response of the *psbDII* gene. *Synechococcus* reporter strains having one or two of the promoter-proximal binding sites of *psbDII* deleted showed a pattern of light induction similar to that of the undeleted control. If the promoter-distal binding site participates in light-regulated expression, point mutations at this site should alter *psbDII* light-responsiveness without eliminating basal expression.

Deletions of the region upstream of the *psbDII-lacZ* transcription start site, including one that extends to nucleotide −42, still permitted a wild-type level of expression and light-induced stimulation of β-galactosidase production. However, expression was abolished when the promoter sequences were deleted. These results, combined with those from the internal deletion experiments, indicate that the upstream promoter element is necessary, but not sufficient, to induce efficient *psbDII* gene expression. Deletion of the three protected regions downstream from the transcription start site resulted in a complete loss of *psbDII* gene expression, even though the 96 bp upstream of the transcription start site remained unaltered. Perhaps sequences controlling the initiation and regulation of *psbDII* transcription compose dual upstream and downstream promoter elements. The upstream element would be located between nucleotide −42 and the transcription start site and include the −10 *E. coli* consensus sequence. The downstream elements may consist of a combination of sequences lying in close proximity to the transcription initiation site. For any significant level of transcription to occur, at least one of the downstream elements may be required. A similar arrangement has been observed in eukaryotic promoters such as those for the ribosomal protein L32 gene (30), the TFIID gene (32), and the mdg1 *Drosophila* retrotransposon (2). In prokaryotes, the *E. coli* LexA repressor binds downstream of the transcription start site to regulate expression of *uvrD*, *cloDF13*, *sulA*, and *cle1-1* (11, 12, 28, 49), although most of these genes contain well-defined and functional upstream promoter regions.

The results presented in this study have localized *cis*-acting regulatory sequences in the untranslated leader region of the *psbDII* gene in *Synechococcus* sp. The β-galactosidase activities associated with different deletion constructs suggest that the *cis*-regulatory elements function as transcriptional activators. The +11 to +84 regulatory region of the *psbDII* gene may comprise three or more *cis*-acting regulatory elements that function either independently or synergistically. Such a scheme has been proposed for gene regulation in mammalian and yeast cells (24), for genes induced by oxidative stress (*oxyR*, *katG*, and *ahpC*) in *E. coli* (46), and for the developmentally expressed *tps* and *ops* genes in *Myxococcus xanthus* (10, 20). The results reported here do not specify the mechanism of *psbDII* regulation by light, but viable possibilities are that control is mediated by the promoter-distal binding site or that induction is achieved through structural changes in proteins already bound to the DNA.

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