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Differential Expression of Members of a Cyanobacterial *psbA* Gene Family in Response to Light

MICHAEL R. SCHAEFER[1] AND SUSAN S. GOLDEN[2]*

Department of Biochemistry and Biophysics[1] and Department of Biology,[2] Texas A&M University, College Station, Texas 77843

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The genome of the cyanobacterium *Synechococcus* sp. strain PCC 7942 contains three *psbA* genes encoding two forms of the D1 protein: form I, the product of *psbAI*, differs from form II, the product of both *psbAII* and *psbAIII*, at 25 of 360 residues. D1 is essential for photosynthesis as a core component of the photosystem II reaction center. Translational gene fusions between each of the *Synechococcus psbA* genes and the *Escherichia coli lacZ* gene were inserted into the chromosome of wild-type *Synechococcus* sp. at the respective *psbA* loci to serve as in vivo reporters of *psbA* expression. β-Galactosidase activities indicated differential expression of the *psbA-lacZ* gene fusions related to light availability. Expression of *psbAI* was 500-fold greater than expression of *psbAII* and 50-fold greater than *psbAIII* under similar conditions. As light intensity decreased from 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 2 $\mu E \cdot m^{-2} \cdot s^{-1}$, expression of the *psbAI* reporter increased eightfold while expression of the *psbAII* and *psbAIII* reporters decreased 10-fold, suggesting differential production of the two forms of D1 in photosystem II in response to light availability. Relative levels of *psbA-lacZ* fusion transcripts directly reflected β-galactosidase activities in the transformants, although the fusion transcripts were less stable than native *psbA* messages.

The thylakoid membrane of cyanobacteria is structurally and functionally similar to that of the chloroplasts of higher plants (8). Both carry out oxygen-evolving photosynthesis, which requires photochemical reactions at two multiprotein complexes, termed photosystems I and II. The reaction center core of photosystem II appears to contain a dimer of two structurally similar proteins, termed D1 and D2, which are encoded by *psbA* and *psbD* genes, respectively (22). Together in the membrane, D1 and D2 house the photoreactive chlorophyll, primary acceptor, and other cofactors involved in photosynthetic electron transport through photosystem II (11, 17, 22). Additionally, D1 is the target of action of several classes of herbicides (23). In higher plants, a single *psbA* gene is located in the chloroplast genome (26) whereas all genera of cyanobacteria examined, including *Anabaena*, *Fremyella*, *Synechococcus*, and *Synechocystis*, possess small *psbA* multigene families (4–6, 9, 16). In the species of *Anabaena* and *Synechococcus* in which all *psbA* genes have been sequenced, two different forms of D1 are encoded that differ slightly in amino acid sequence (4, 6; J. M. Vrba and S. E. Curtis, personal communication).

We are interested in the functional significance of maintaining multiple *psbA* genes and expressing two forms of D1 in *Synechococcus* sp. strain PCC 7942 (hereafter referred to as *Synechococcus*). The chromosome of *Synechococcus* contains three distinct copies of the *psbA* gene. Extensive characterization has shown that all three genes are monocistronic, transcriptionally active, and individually capable of supporting photoautotrophic growth (6). The *psbA* multigene family encodes two forms of D1; form I (the product of *psbAI*) differs from form II (the product of both *psbAII* and *psbAIII*) at 25 residues, 12 of which are in the first 16 amino acids of the protein as predicted by the three 1,080-base-pair *psbA* genes (6).

An efficient DNA transformation system for *Synechococcus* makes it possible to insert modified alleles of specific genes into the chromosome as gene duplications at their native loci (7). We constructed translational gene fusions between the individual *psbA* genes and an *Escherichia coli lacZ* gene to serve as in vivo reporters of *psbA* expression. By established methods, each of the *psbA-lacZ* translational gene fusions was inserted into the chromosomes of wild-type cells. Expression of β-galactosidase in strains containing the *psbA-lacZ* gene fusions indicated that the *psbA* genes in *Synechococcus* are differentially expressed and suggested that light availability may affect the ratio of D1 forms in photosystem II through altered expression of the different *psbA* genes.

MATERIALS AND METHODS

Strains. Wild-type and mutant strains of *Synechococcus* sp. (previously referred to as *Anacystis nidulans* R2, Pasteur Culture Collection no. 7942) were grown in liquid BG-11 medium (1) or on solid BG-11 agar as previously described (7). Mutant strains harboring selectively inactivated *psbA* genes have been described (6). A detailed protocol for DNA-mediated transformation of this strain has been published (7). Mutant and transformed strains of *Synechococcus* were cultured in the presence of one or more of the following selective agents (in micrograms $milliliter^{-1}$): kanamycin sulfate (50), spectinomycin (40), chloramphenicol (7.5), and ampicillin (0.5) (Polycillin N; Bristol Laboratories, Syracuse, N.Y.).

E. coli strain DH5α, purchased from Bethesda Research Laboratories, Gaithersburg, Md., was the host for all plasmids. The following antibiotics (in micrograms $milliliter^{-1}$) were added to LB growth medium (GIBCO Diagnostics, Madison, Wis.) (10) for selection of plasmids in *E. coli*: ampicillin (100), chloramphenicol (17), kanamycin sulfate (50), spectinomycin (25), and tetracycline (12.5).

DNA and RNA probe methods. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 DNA kinase, calf intestinal alkaline phosphatase, mung bean nuclease, and S1 nuclease were purchased from Bethesda Research Labora-

* Corresponding author.

tories; New England BioLabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; or Promega Biotec, Madison, Wis. DNA manipulations including restriction digests, agarose gel electrophoresis, dephosphorylation, ligation, and hybridization of Northern (RNA) blots were carried out as described by Maniatis et al. (10) or Ausubel et al. (2). For Southern blot hybridization analysis, DNA was transferred to a charged nylon membrane (GeneScreen Plus, New England Nuclear Research Products, or Magnagraph, Micron Separations Inc.) by the alkaline capillary method of Reed and Mann (19) and was hybridized according to the recommendations of the manufacturers. Radioactive DNA probes were produced from gel-purified DNA fragments (25) either by nick translation or random primer labeling with kits from Bethesda Research Laboratories.

Total RNA was isolated from *Synechococcus* as described by Golden et al. (7) with a minor modification. Pelleted cells were suspended in 5 ml of a 15:1 mixture of 50/100 TE (50 mM Tris, 100 mM EDTA [pH 8.0]) and vanadyl ribonucleoside complex (10) and frozen at −90°C overnight. Upon thawing, 1.3 ml of 20% sodium dodecyl sulfate and 5 ml of glass beads were added to the suspension and RNA was isolated by using the reported protocol. For Northern blot analysis, RNA was denatured with formaldehyde, separated by gel electrophoresis on a 1.2% agarose gel, and transferred to a charged nylon membrane as described by Ausubel et al. (2).

Radioactive anti-sense RNA probes were produced from pT7T3 plasmids (Bethesda Research Laboratories) containing unique upstream regions of the *psbA* genes by transcription with T7 polymerase (3a). Northern blot filters were hybridized at 50°C in a mixture of 50% formamide, 5× SSPE (10), and 1% sodium dodecyl sulfate and were washed at 65°C in 0.5× SSPE–0.1% sodium dodecyl sulfate. The 5′ ends of *psbA-lacZ* fusion transcripts were mapped by using mung bean nuclease or S1 nuclease protection experiments. Reactions contained 10 or 50 μg of RNA and 40,000 cpm of 5′-end-labeled DNA as described by Tumer et al. (24). The protected fragments were resolved on polyacrylamide-urea gels and measured against A+G sequencing ladder markers (13).

Translational gene fusions and plasmids. Plasmids pDH140, pSG201, and pSG302 have been described (6). These plasmids are pBR328 derivatives containing the entire *psbAI* gene or truncated *psbAII* or *psbAIII* genes; *psbAII* and *psbAIII* are lethal to *E. coli* when cloned intact (6). To construct the Φ(*psbAI-lacZ*)(Hyb) translational gene fusion (hereafter designated *psbAI-lacZ*), pDH140 was cleaved with *Bst*EII, which cuts within the *psbAI* gene at nucleotides 60 and 554 of the open reading frame. The unpaired bases were repaired with polymerase I Klenow fragment to leave a blunt end after the second nucleotide of *psbAI* codon 22. A truncated *lacZ* gene was removed from plasmid pMC1871 (21) by digestion with *Sal*I, and unpaired bases were repaired as described for pDH140. The resulting blunt fragment supplied the third base for codon 22 and fused the remainder of *lacZ* in frame with *psbAI*. The internal *Bst*EII fragment from *psbAI* was removed during the construction of this plasmid, which was designated pAM129. Constructions fusing a truncated *lacZ* gene in frame with *psbAII* (pAM017) and *psbAIII* (pAM019) were based on *Bst*EII digestion of plasmids pSG201 and pSG302, respectively, and *Sal*I digestion of pMC1871 to produce the truncated *lacZ* fragment; for these fusions, blunt ends were generated by using mung bean nuclease rather than DNA polymerase I (Klenow fragment). Therefore, the Φ(*psbAII-lacZ*)(Hyb) and Φ(*psbAIII-lacZ*) (Hyb) translational gene fusions (hereafter designated *psbAII-lacZ* and *psbAIII-lacZ*, respectively) encode the first 20 amino acids of each *psbA* gene product fused in frame with β-galactosidase. In-frame insertion of *lacZ* was confirmed for the three translational gene fusions by sequencing the *psbA-lacZ* junctions (20). Plasmid pAM017 was modified for transformation of *Synechococcus* by insertion of the 2-kilobase (kb) streptomycin-spectinomycin resistance cassette from pHP45Ω (18) into a unique *Pst*I site, forming pAM309.

Assay culture conditions. *Synechococcus* strains were cultured at 30°C in glass carboys containing 8 liters of BG-11 for the experiments shown in Fig. 6 and 7 (see below). Light was provided by four 18-in. (45.72 cm) 15-W tubes placed 4 cm from the culture vessel. The standard inoculum was equivalent to a 5-ml cell culture with unit absorbance due to light scattering at 750 nm (A_{750} = 1.0). The cultures were continuously stirred and bubbled with 1% CO_2 in air. As the cultures matured to stationary phase, successive samples containing equivalent cell mass were removed for determination of optical density, soluble protein, and β-galactosidase activity. At each sampling, photosynthetic photon flux density (PPFD; photosynthetically active radiation between 400 and 700 nm, expressed as $\mu E \cdot m^{-2} \cdot s^{-1}$) was measured at the center of the culture with an ethanol-sterilized immersible probe (Biospherical Instruments, Inc., San Diego, Calif.).

Protein and β-galactosidase assays. Cells were harvested by centrifugation and suspended in 1 ml of a mixture of cold 10 mM Tricine (pH 7.5), 0.005% sodium dodecyl sulfate, 5 mM ε-aminocaproic acid, 1 mM benzamidine, and 80 μM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). After the addition of 0.5 ml of chloroform, the suspensions were vortexed for 1 min to lyse the cells. Portions from the aqueous phase were removed for determination of soluble protein by a modified Lowry procedure (12). β-Galactosidase activity in the aqueous phase was determined by colorimetric assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) according to Miller (15) and expressed as specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside $\cdot$ min^{-1} $\cdot$ mg of $protein^{-1}$.

RESULTS

Recombining *psbA-lacZ* reporter genes into the *Synechococcus* genome. Steady-state expression of the *psbA* multigene family in *Synechococcus* was measured in vivo by using translational gene fusions between the individual *psbA* genes and the *E. coli lacZ* gene as biochemical reporters of *psbA* expression. The *psbA-lacZ* gene fusions were constructed on nonreplicating plasmids containing entire or partial *psbA* genes from *Synechococcus* (6). For each gene, a *lacZ* coding region lacking the first eight amino-terminal codons (21) was inserted in frame into each *psbA* coding region at a common *Bst*EII site located 60 base pairs downstream of the translational start site (as described in Materials and Methods). By this strategy, in vivo expression of a chromosomally inserted *psbA-lacZ* gene fusion was under control of *psbA* transcription and translation signals, producing a soluble fusion protein consisting of the first 20 or 22 amino-terminal residues of D1 followed by a functional β-galactosidase subunit. The unique amino termini of the two forms of D1 are, thus, the only difference between the two fusion proteins encoded by the three *psbA-lacZ* gene fusions. Each of three plasmids containing specific *psbA-lacZ* gene fusions (Table 1) was

TABLE 1. *Synechococcus* strains used to analyze *psbA* expression

Strain	Integrated plasmid	*psbA-lacZ* gene fusion	Relative location[a]	Antibiotic resistance[b]
PCC 7942	None	None		None
AMC051	pAM129	*psbAI-lacZ*	Upstream	Cm,Ap
AMC052	pAM309	*psbAII-lacZ*	Upstream	Sp
AMC053	pAM019	*psbAIII-lacZ*	Upstream	Cm
AMC066	pAM129	*psbAI-lacZ*	Downstream	Cm,Ap
AMC067	pAM019	*psbAIII-lacZ*	Downstream[c]	Cm

[a] Location on chromosome relative to respective *psbA* locus.
[b] Antibiotic concentrations (in micrograms milliliter^{-1}) were as follows: chloramphenicol, 7.5; ampicillin (Polycillin N), 0.5; spectinomycin, 40.
[c] Native *psbAIII* gene nonfunctional.

used to transform wild-type cells by established methods for transformation of *Synechococcus* (7). All transformants exhibited assayable β-galactosidase activity and were designated Lac$^+$.

Strains AMC051 and AMC066 were isolated by transforming wild-type cells with plasmid pAM129, which carries the *psbAI-lacZ* gene fusion, and selecting for resistance to chloramphenicol and ampicillin. Both strains exhibited β-galactosidase activity. In strain AMC051, a single homologous recombination event occurred between the chromosomal *psbAI* locus and pAM129 upstream of the *psbAI-lacZ* gene fusion (Fig. 1). This resulted in the insertion of pAM129 DNA into the chromosome, placing the *psbAI-lacZ* gene fusion upstream of heterologous vector sequences from pAM129. In this orientation, the *psbAI-lacZ* gene fusion resides almost immediately upstream of the intact *psbAI* gene in an otherwise wild-type genetic background, and transcription from the two *psbAI* promoters is in the same direction. Strain AMC066 resulted from a single homologous recombination event between downstream DNA sequences. In this strain, the native *psbAI* gene is upstream of and in the same orientation as the *psbAI-lacZ* gene fusion.

Integration of the *psbAII-lacZ* and *psbAIII-lacZ* gene fusions into the chromosome was achieved by transforming wild-type cells with plasmids pAM309 and pAM019, respectively. Strain AMC052 contains the *psbAII-lacZ* gene fusion upstream of the intact *psbAII* gene at that locus. Similarly, strain AM053 contains the *psbAIII-lacZ* gene fusion upstream of the *psbAIII* gene. The starting plasmid for construction of the *psbAIII-lacZ* gene fusion, pSG302, contained a *Pst*I fragment extending from 1.2 kb upstream of the open reading frame to +464 base pairs into the *psbAIII* coding region. The product of a downstream homologous recombination was a strain (AMC067) containing pAM019 DNA inserted immediately downstream of the *psbAIII* gene. However, the *psbAIII* gene in strain AMC067 was nonfunctional because of the truncated *psbAIII* open reading frame on plasmid pAM019. Plasmid pAM309 contained a truncated *psbAII* gene as part of the *psbAII-lacZ* gene fusion, but no transformants containing a similar downstream insertion of plasmid DNA were isolated.

The insertion of plasmid DNA at the expected loci in strains which contain *psbA-lacZ* gene fusions was confirmed by Southern blot analysis (Fig. 2). Genomic DNA from wild-type and Lac$^+$ transformants was digested with *Eco*RI

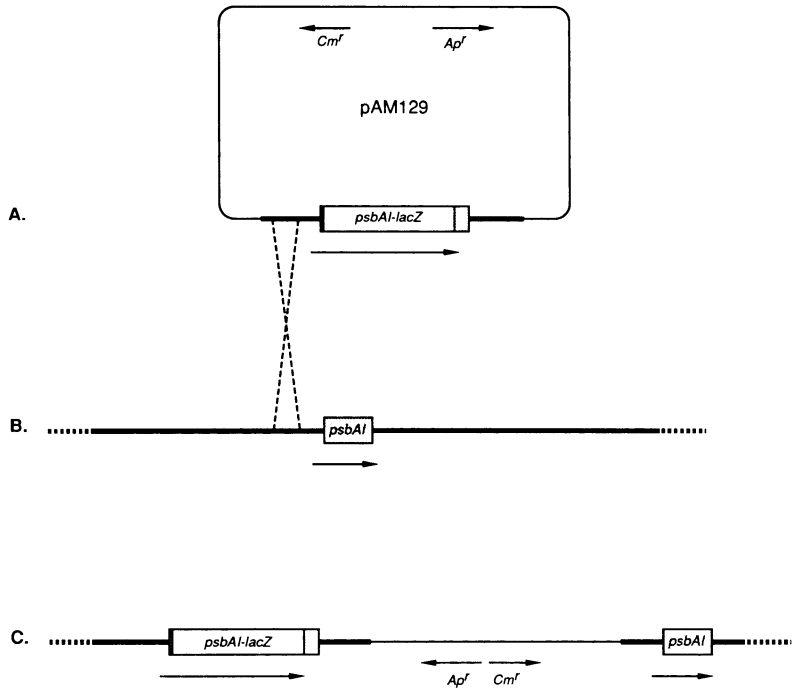

FIG. 1. Insertion of the *psbAI-lacZ* translational gene fusion into the chromosome of wild-type *Synechococcus* upstream of *psbAI*. Open boxes represent the truncated *E. coli lacZ* coding region and stippled boxes represent *psbAI* coding sequences. Heavy and thin lines depict *Synechococcus* and vector DNA, respectively. Arrows denote direction of transcription. Broken lines depict a single homologous recombination event between DNA upstream of the *psbAI-lacZ* gene fusion on pAM129 (A, not drawn to scale) and the *psbAI* locus (B), resulting in insertion of plasmid DNA immediately upstream of the native *psbAI* gene on the chromosome (C). The transformed strain, AMC051, contains the *psbAI-lacZ* gene fusion upstream of heterologous plasmid DNA and the intact *psbAI* gene (C, drawn to scale) with transcription from both *psbAI* promoters in the same direction. Location and direction of transcription of genes encoding resistance to chloramphenicol (Cm^r) and ampicillin (Ap^r) are also depicted.

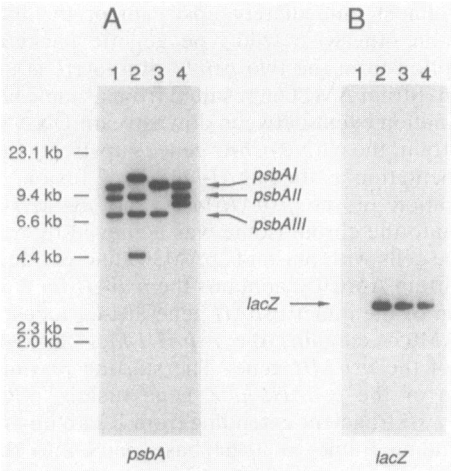

FIG. 2. Southern blot analysis of DNA from Lac$^+$ strains. Total DNA was isolated from wild-type *Synechococcus* and from strains containing each of the *psbA-lacZ* translational gene fusions. The DNA was cut with *Eco*RI, separated by agarose gel electrophoresis, transferred to a nylon membrane, and sequentially hybridized with probes recognizing *psbA* or *lacZ* sequences. (A) Southern blot hybridized with the *psbA* probe. (B) Southern blot shown in panel A after stripping *psbA* probe and hybridization with the *lacZ* probe. The *psbA* probe was a 1-kb *Dde*I fragment containing the *psbAI* open reading frame that recognizes all three *psbA* genes. The *lacZ* probe was a 3.0-kb *Eco*RI fragment of pMC1871 containing most of the *lacZ* open reading frame. DNA from the following strains are indicated: wild type (lanes 1); AMC051 (lanes 2); AMC052 (lanes 3); AMC053 (lanes 4). Arrows indicate location of wild-type *psbA* fragments (A) and the *lacZ* fragment (B).

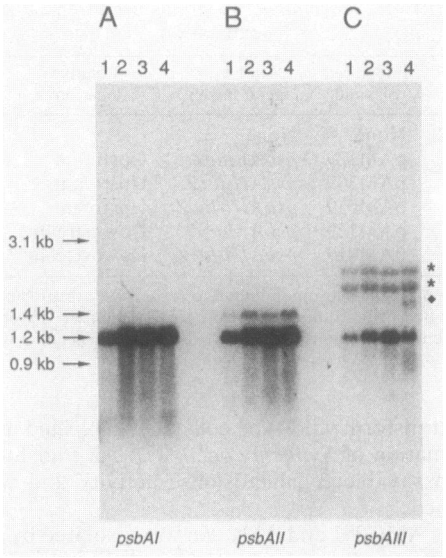

FIG. 3. Northern blot analysis of RNA from each of the *psbA* genes in the Lac$^+$ strains. Total RNA was isolated from wild-type *Synechococcus* and from strains containing each of the *psbA-lacZ* translational gene fusions. Triplicate RNA samples were denatured with formaldehyde, separated by agarose gel electrophoresis, and transferred to nylon filters, and each set of lanes was hybridized with a different gene-specific antisense RNA probe. Represented are Northern blots hybridized with *psbAI*-specific probe (A), *psbAII*-specific probe (B), and *psbAIII*-specific probe (C). Samples (5 μg) of total RNA from the following *Synechococcus* strains are indicated: wild type (lanes 1); AMC051 (lanes 2); AMC052 (lanes 3); AMC053 (lanes 4). Symbols: ★, 23S rRNA and its characteristic stable breakdown product; ◆, unidentified RNA species in strain AMC053 detected with the *psbAIII*-specific probe.

or *Bam*HI and hybridized with probes for *psbA* or *lacZ*. With the *psbA* probe, the three *psbA* genes in wild-type cells were identified as *Eco*RI fragments of 11.0, 9.0, and 7.0 kb containing *psbAI*, *psbAII*, and *psbAIII*, respectively (Fig. 2A, lane 1). Lanes 2 through 4 of Fig. 2A contain *Eco*RI-digested genomic DNA from the Lac$^+$ strains and show that insertion of plasmid DNA at each locus alters the length (and sometimes number) of fragments associated with the respective gene. For example, the insertion of pAM129 DNA at the *psbAI* locus of strain AMC051 increased the size of the *Eco*RI fragment containing *psbAI* from 11.0 to approximately 12.2 kb (Fig. 2A, lane 2). This 1.2-kb increase closely matches the predicted fragment length associated with an upstream insertion of plasmid DNA at the *psbAI* locus. The altered fragments for strains AMC052 and AMC053 (Fig. 2A, lanes 3 and 4) similarly match predicted changes following upstream insertions of plasmid DNA at the respective loci. More precise maps of the recombinant chromosomes in the Lac$^+$ strains were confirmed on Southern blots which contained DNA digested with *Bam*HI (data not shown). Hybridization of the same *Eco*RI-digested DNA blot with a *lacZ* probe shows that all three Lac$^+$ strains contain the 3.0-kb *lacZ* fragment (Fig. 2B, lanes 2 through 4).

Transcription of wild-type *psbA* genes and the respective *psbA-lacZ* gene fusions in each of the Lac$^+$ strains was examined by Northern blot analysis (Fig. 3). Gene-specific antisense RNA probes were used to detect *psbA* and *psbA-lacZ* transcripts on blots containing total RNA from wild-type and each of the transformed strains. With probes specific for *psbAI*, *psbAII*, and *psbAIII*, native 1.2-kb *psbA* transcripts were identified in all four strains (Fig. 3A, B, and C, lanes 1 through 4, respectively). However, the gene-specific RNA probes did not detect predicted 3.1-kb transcripts from *psbAI-lacZ* in strain AMC051 (Fig. 3A, lane 2), *psbAII-lacZ* in strain AMC052 (Fig. 3B, lane 3), or *psbAIII-lacZ* in strain AMC053 (Fig. 3C, lane 4). In addition to the 1.2-kb *psbAII* transcript, a 1.4-kb RNA species was identified by the *psbAII*-specific probe (Fig. 3B, lanes 1 through 4) as previously identified by J. A. Brusslan and R. Haselkorn (personal communication). Initial studies have suggested that this larger transcript arises from a second promoter for *psbAII* upstream of the previously identified transcriptional start site (S. A. Bustos and S. S. Golden, unpublished data). The *psbAIII*-specific probe cross-hybridized with two species of 23S rRNA in RNA from all strains (Fig. 3C, lanes 1 through 4) and with an unidentified 1.6 kb-RNA species in strain AMC053 (Fig. 3C, lane 4).

In order to confirm the presence of *psbA-lacZ* transcripts in the three Lac$^+$ strains, a Northern blot containing total RNA isolated from cells harvested during rapid or stationary growth phase was hybridized with *psbAI* and *lacZ* probes (Fig. 4). The RNA from wild-type *Synechococcus* was obtained only from cells at stationary phase. The *psbAI* probe should hybridize with all three native *psbA* transcripts, thus providing an indication of total *psbA* transcript abundance at the two growth phases (Fig. 4A). During rapid growth, the three Lac$^+$ strains appeared to have similar levels of *psbA* mRNA (Fig. 4A, lanes 2, 4, and 6) and exhibited equal reduction in transcript quantity relative to total RNA during stationary growth (Fig. 4A, lanes 3, 5, and 7). The *psbAI* probe also detected the previously reported 0.9-kb incomplete *psbAI* mRNA (6).

Hybridization of the Northern blot with the *lacZ* DNA probe did not detect full-length *psbA-lacZ* fusion transcripts

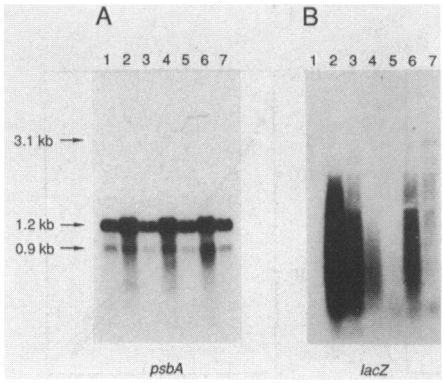

FIG. 4. Northern blot analysis of *psbA* and *psbA-lacZ* transcripts in Lac$^+$ strains. Total RNA was isolated from wild-type *Synechococcus* and strains containing each of the *psbA-lacZ* translational gene fusions. For the Lac$^+$ strains, RNA was extracted from cells at rapid (A and B, lanes 2, 4, and 6) and stationary (A and B, lanes 3, 5, and 7) phases of growth. The RNA was denatured with formaldehyde, separated by agarose gel electrophoresis, transferred to a nylon filter, and hybridized with DNA probes recognizing *psbA* or *lacZ* as described in the legend to Fig. 2. (A) Northern blot hybridized with the *psbA* probe. (B) Northern blot shown in A after stripping *psbA* probe and hybridizing with the *lacZ* probe. Samples (5 μg) of total RNA from the following *Synechococcus* strains are indicated: wild type (lanes 1); AMC051 (lanes 2 and 3); AMC052 (lanes 4 and 5); AMC053 (lanes 6 and 7).

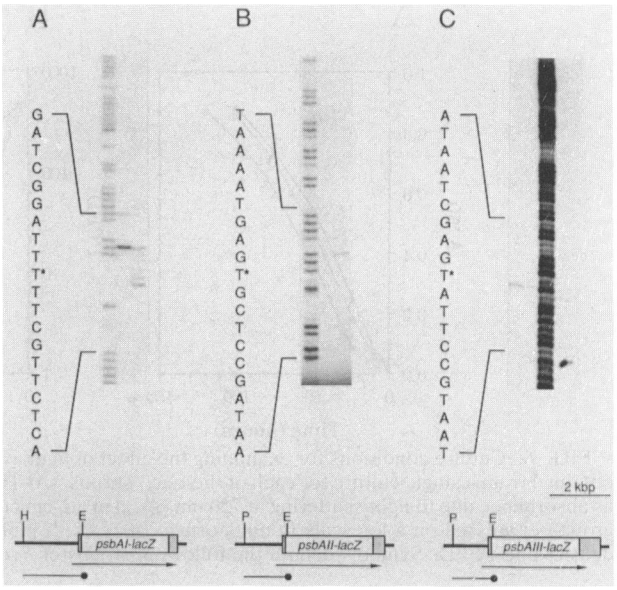

FIG. 5. Mapping 5′ ends of *psbA-lacZ* transcripts by nuclease protection. Total RNA isolated from each of the Lac$^+$ strains was annealed to a denatured 5′-end-labeled DNA fragment from the respective *psbA-lacZ* gene fusion, and the heteroduplexes were digested with either mung bean or S1 nuclease. The products of each digestion were resolved on sequencing gels alongside products of Maxam and Gilbert A+G sequencing reactions of the respective end-labeled DNA fragments (13). Reaction products are shown for transcripts from strains AMC051 (A), AMC052 (B), and AMC053 (C). For autoradiographs in A and B, the A+G sequencing ladder is in the left lane and mung bean nuclease-protected fragments are to the right. For the autoradiograph in C, the A+G sequencing ladder is in the center lane with a mung bean nuclease-protected fragment at the left and S1 nuclease-protected fragments at the right. The end-labeled fragments for mapping the three *psbA-lacZ* fusion transcripts were generated by the digestion of plasmids pAM129, pAM309, and pAM019 with *Eco*RI and the labeling of that site, producing fragments with labeled 5′ ends within the *lacZ* portion of each gene fusion and extending upstream to secondary cleavage sites (schematically represented at the bottom). The end-labeled fragments were obtained by secondary cleavage with *Hin*dIII for *psbAI-lacZ* and *Pst*I for *psbAII-lacZ* and *psbAIII-lacZ*. The mung bean nuclease protection experiments produced a group of bands at the 5′ ends of the *psbAI-lacZ* and *psbAII-lacZ* transcripts. ★, 5′ Start sites determined for native *psbA* transcripts in *Synechococcus* (6).

in any of the three Lac$^+$ strains (Fig. 4B). As expected, the *lacZ* probe did not hybridize to RNA from wild-type cells (Fig. 4B, lane 1), but various degrees of hybridization were detected as signal smearing in lanes containing RNA from the Lac$^+$ strains (Fig. 4B, lanes 2 through 7). These smears were interpreted as confirmation of transcription of the *psbA-lacZ* gene fusions; however, it was apparent that the fusion transcripts were much less stable than native *psbA* transcripts in *Synechococcus*. The relative signal intensities on the blot directly reflected the relative levels of β-galactosidase activity among the Lac$^+$ strains (see below). During the phase of rapid growth, the abundance of *psbAI-lacZ* mRNA appeared to be much greater than that of either *psbAII-lacZ* or *psbAIII-lacZ* (Fig. 4B, lanes 2, 4, and 6, respectively). It was also apparent that under these conditions, the steady-state level of *psbAIII-lacZ* mRNA was significantly greater than that of *psbAII-lacZ*. Comparison of signal intensities among the strains during rapid and stationary growth indicated that overall *psbA-lacZ* transcription decreased during stationary growth and that for the two conditions examined the ratio of the three *psbA-lacZ* transcript levels remained fairly constant (Fig. 4B, lanes 2, 4, and 6 versus lanes 3, 5, and 7).

In order to ensure that transcription of the *psbA-lacZ* gene fusions was initiating at the respective *psbA* start sites, the 5′ ends of the transcripts were mapped by nuclease protection experiments. The protection substrate had a labeled 5′ end originating within the *lacZ* cassette so that native *psbA* messages would not provide protection (Fig. 5). In RNA from strains AMC051 and AMC052, the 5′ ends of the respective *psbA-lacZ* transcripts were mapped to within two bases of the 5′ ends reported for wild-type *psbAI* and *psbAII* transcripts (6) by using mung bean nuclease (Fig. 5A and B, respectively). Similarly, S1 nuclease was used to map the 5′ end of the *psbAIII-lacZ* fusion transcript in strain AMC053 to the published 5′ end of the *psbAIII* transcript (6) (Fig. 5C). Thus, although the messages from the reporter genes were relatively less stable than those of their wild-type counterparts, they resulted from activity of the respective *psbA* promoters.

β-Galactosidase activity in the Lac$^+$ strains. β-Galactosidase specific activities in lysates from strains AMC051 and AMC053 indicated that expression of *psbAI* was much greater than that of *psbAIII* under standard growth conditions. Further examination of these two strains revealed that after transfer of 100-ml cultures to conditions of lower light intensity, β-galactosidase activity in strain AMC051 increased twofold while that of strain AMC053 decreased sharply, suggesting differential expression of the *psbA* multigene family as a function of light availability. In order to study the relationship between *psbA* expression and light availability in *Synechococcus*, large-volume cultures of strains AMC051, AMC052, and AMC053 were maintained under controlled environmental conditions (as described in Materials and Methods). After inoculation, individual cul-

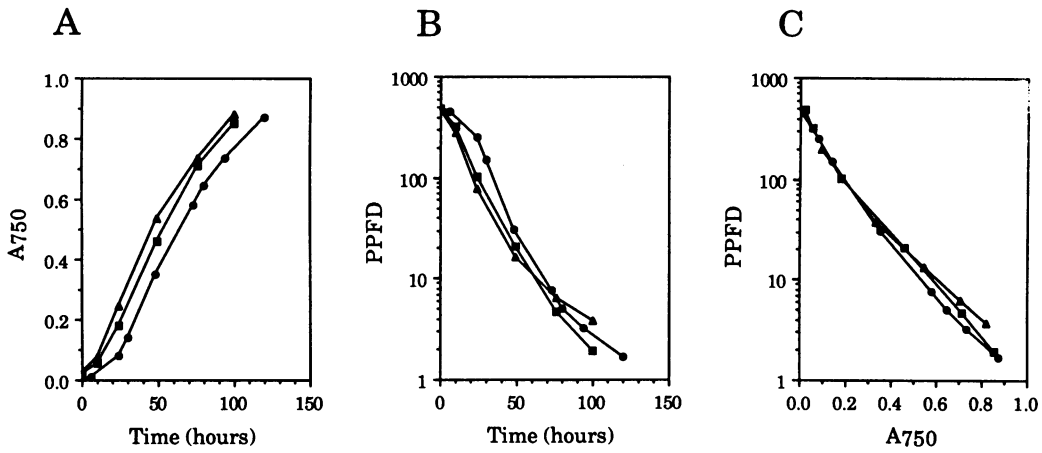

FIG. 6. Culture conditions for examining the effect of light availability on *psbA* expression in *Synechococcus*. Each curve represents data obtained from a single culture for each of the Lac$^+$ strains. (A) Time course of culture development. Increasing culture cell density, expressed as absorbance due to light scattering at 750 nm (A_{750}) in a 1-cm cuvette, was plotted as a function of time. (B) Decreasing PPFD during culture growth expressed on a log scale in units of $\mu E \cdot m^{-2} \cdot s^{-1}$, plotted as a function of time. (C) The inverse relationship between culture cell density and PPFD. Symbols denote the following strains of *Synechococcus*: ●, AMC051; ■, AMC052; ▲, AMC053.

tures of the three Lac$^+$ strains exhibited similar growth rates and typically took approximately 100 h to reach the initial stages of stationary growth (Fig. 6A). As the culture cell density increased with time, PPFD measured directly from the center of the culture decreased from nearly 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 2 $\mu E \cdot m^{-2} \cdot s^{-1}$ because of photosynthetic absorbance by the cells (Fig. 6B). The decrease in PPFD was directly related to cell density (Fig. 6C), and, therefore, nearly logarithmic as measured in the center of a culture bottle. It is important to note that at a given moment, a gradient of low-to-high PPFD was present from the center of the culture outward toward the light source and that measured PPFD represented the low extreme at the respective cell density. Furthermore, as cell density increased, the PPFD gradient sharply increased. Since the cultures were constantly mixed, measured PPFD was used as a representative standard of light availability at each determination.

Experiments with the Lac$^+$ strains indicated that expression of *psbAI* was high and inversely related to light availability. As PPFD decreased from 600 to 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ in strain AMC051, β-galactosidase specific activity exhibited an eightfold increase from 190 to 1,500 U (Fig. 7A). At PPFD below 20 $\mu E \cdot m^{-2} \cdot s^{-1}$, expression of *psbAI* appeared to remain high and constant, suggesting a greater need for form I of the D1 protein in a low-light environment. Under the same conditions, expression of both *psbAII* and *psbAIII* was low and directly related to light availability (Fig. 7B and C, respectively). In contrast to that in strain AMC051, β-galactosidase activity in strains AMC052 and AMC053 decreased 10-fold as PPFD decreased from 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 7B and C, respectively). The effect of changing PPFD on expression of both *psbAII* and *psbAIII* was identical, with expression of *psbAIII* approximately 10-fold that of *psbAII* along the standard gradient of light. At a standard PPFD of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, expression of *psbAI* was 500-fold greater than that of *psbAII* and 50-fold greater than that of *psbAIII*. Two experiments have indicated that the changes in expression of *psbAI* are responses

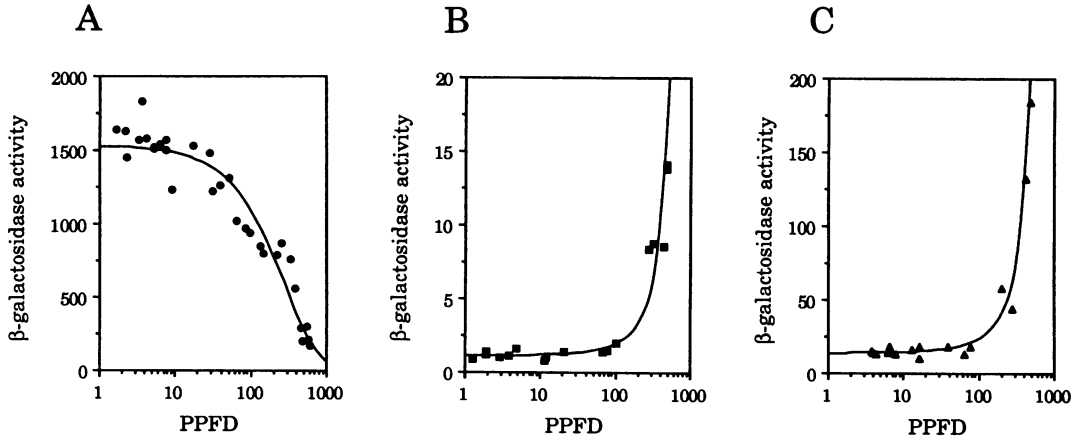

FIG. 7. Differential expression of the *psbA* multigene family in *Synechococcus* related to light availability. Expression of *psbAI-lacZ* (A), *psbAII-lacZ* (B), and *psbAIII-lacZ* (C) gene fusions based on β-galactosidase specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside $\cdot$ min^{-1} $\cdot$ mg of $protein^{-1}$) was determined along the experimental time course and was plotted as a function of culture PPFD ($\mu E \cdot m^{-2} \cdot s^{-1}$). Note that the scale for β-galactosidase activity is different for each plot. All data points derived from three replicate experiments are shown. Superimposed on each plot is a computer-generated curve representing an exponential best fit with >93% confidence limits for the data. PPFD is plotted on a log scale of low-to-high intensity as read from left to right. Symbols denote the following strains of *Synechococcus*: ●, AMC051; ■, AMC052; ▲, AMC053.

to light intensity rather than to culture age or cell density. When two cultures of AMC051 were inoculated identically but illuminated at 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ or 500 $\mu E \cdot m^{-2} \cdot s^{-1}$, β-galactosidase activity was 40% higher in the lower-light culture; however, cell density was lower in the lower-light culture since *Synechococcus* growth rate is light dependent. In both cultures, β-galactosidase activity was strongly correlated with PPFD, not with cell density. In another study, 50-ml samples from a culture illuminated at 330 $\mu E \cdot m^{-2} \cdot s^{-1}$ were transferred to different light intensities. After 2 h, β-galactosidase activity in a sample illuminated at the same intensity as in the original culture was unchanged (550 U). However, a sample at 660 $\mu E \cdot m^{-2} \cdot s^{-1}$ decreased 2% in activity while a sample at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ increased 36% in activity.

Pulse-chase analysis of total soluble proteins from strains AMC051 and AMC053 labeled with ^{35}S indicated approximately equal rates of degradation between the two forms of D1-β-galactosidase hybrid protein (data not shown). Thus, differences in β-galactosidase activities produced by the three gene fusions were not caused by a difference in protein stability conferred by the unique amino-terminal domains.

DISCUSSION

The *psbA-lacZ* translational gene fusions served as effective reporters of *psbA* expression in *Synechococcus*. We assume that β-galactosidase specific activity in strains containing *psbA-lacZ* translational gene fusions reflected steady-state expression of the respective *psbA* gene. In the Lac^+ strains reported here, the *psbA-lacZ* gene fusions were inserted into the chromosome as gene duplications, leaving intact the respective *psbA* gene, except in AMC067, in which the native *psbAIII* gene was truncated. This strategy was used to maintain the presence of both wild-type gene products in the cell. Removing one of the forms of D1 by replacing the *psbAI* gene might significantly alter the observed regulatory pattern. Comparison of β-galactosidase activities in strains AMC053 and AMC067 support such a regulatory mechanism for *psbAIII*. Assuming equal expression from a native *psbA* gene and its respective *psbA-lacZ* gene fusion in a transformed strain, levels of β-galactosidase activity should have reflected 50% of total *psbA* expression for the locus. Indeed, β-galactosidase specific activity in strain AMC067, which contains a truncated *psbAIII* gene, was twice that measured for strain AMC053, in which the native *psbAIII* gene was functional (data not shown); however, the wild-type *psbAIII* promoter in strain AMC067 remained intact. This observation argues against titration of a regulatory factor by an increased number of *psbAIII* promoters and supports a regulatory mechanism based on abundance of the *psbAIII* gene product.

The possible influence that orientation of the reporter gene relative to the native *psbA* gene might have on gene expression can be examined only for the *psbAI-lacZ* transformants. In strain AMC051, which contains the *psbAI-lacZ* gene fusion upstream of the *psbAI* gene, β-galactosidase specific activity under standard conditions was within 5% that of strain AMC066, in which the *psbAI-lacZ* gene fusion resides downstream of the *psbAI* gene (data not shown). Both strains AMC051 and AMC066 exhibit identical β-galactosidase responses to decreasing light availability, which suggests that location of the *psbA-lacZ* gene fusion relative to the respective *psbA* gene does not significantly affect expression from either gene.

The individual genes of the *psbA* multigene family in *Synechococcus* are differentially expressed in wild-type cells under normal culture conditions. Based on relative β-galactosidase specific activities in the Lac^+ strains under standard conditions, expression of *psbAI* was much greater than that of both *psbAII* and *psbAIII*. Additionally, expression of *psbAIII* was 10-fold greater than expression of *psbAII*; thus, the relative expression of the *psbA* multigene family in *Synechococcus* is *psbAI* $\gg$ *psbAIII* $>$ *psbAII*. Higher expression of *psbAI* relative to both *psbAII* and *psbAIII* was also reflected in *psbA-lacZ* transcript levels based on signal intensities in Northern blot analysis of total RNA from the Lac^+ strains. These data are in close agreement with earlier work which showed that of every 100 *psbA* transcripts, 94 were from *psbAI*, 1.4 were from *psbAII*, and 4.6 were from *psbAIII* (6). In that study, gene-specific probes were used to quantitate relative *psbA* transcript levels in total RNA isolated from wild-type cells cultured under relatively low light. More recently, a systematic quantitation of *psbA* transcript levels in wild-type *Synechococcus* was performed using RNA isolated from cells harvested at late stationary phase (3a). In that study, total RNA was protected from nuclease degradation by annealing gene-specific antisense RNA probes from upstream untranslated regions of the respective *psbA* transcripts; this showed *psbAI* mRNA to represent 91.2% of total *psbA* transcripts, with *psbAII* and *psbAIII* contributing 5.9% and 2.9%, respectively. The increased proportion of *psbAII* transcripts relative to previous measurements (6) and our data was attributed to an up mutation in *psbAII* during laboratory culture (3a).

At present, the significance of greater expression of *psbAI* relative to *psbAII* and *psbAIII* is not clear. In comparing putative promoter regions among the three *psbA* genes, *psbAII* and *psbAIII* are preceded by *E. coli*-like consensus sequences, whereas the putative promoter of *psbAI* lacks a conserved −10 sequence (6). This is supported by the fact that the *psbAI-lacZ* fusion, which is highly expressed in *Synechococcus*, exhibits a white colony phenotype in *E. coli* on plates containing the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Whether the *psbAI* promoter constitutes a strong promoter in *Synechococcus* or whether specific gene-activating factors enhance expression of *psbAI* is currently under investigation.

In *Synechococcus*, expression of the individual *psbA* genes correlated with light availability. As PPFD decreased from a maximum of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 20 $\mu E \cdot m^{-2} \cdot s^{-1}$, expression of *psbAI* increased eightfold while expression of both *psbAII* and *psbAIII* decreased 10-fold. Several lines of evidence show that the increase in β-galactosidase specific activity in strain AMC051 is caused by light intensity and not by accumulation of a stable enzyme in a maturing culture. The strongest support for light-mediated expression comes from light-shift studies with strain AMC051 discussed in the previous section. Similar investigations with strains AMC052 and AMC053 are limited by the narrow range of PPFD affecting expression under our culture conditions. If the reporter protein for *psbAI* was much more stable than the identical reporter protein for *psbAII* and *psbAIII*, differential expression between the two gene types could be an artifact. Bachmair et al. (3) have reported that in *Saccharomyces cerevisiae*, the half-life of a β-galactosidase fusion protein is a function of its amino-terminal residue. In our system, both forms of β-galactosidase fusion proteins have the same amino-terminal dipeptide methionine-threonine. It has been shown in spinach that the methionine residue of D1 is cleaved during maturation, leaving *N*-acetyl-*O*-phosphothreonine as the amino-terminal amino acid (14). Preliminary pulse-chase experiments indicate that the two forms of

psbA-lacZ fusion products are equally turned over under similar conditions, although both appear relatively stable in comparison with host soluble proteins (data not shown). If they are equally stable, the decrease in activity with increasing cell density in strains AMC052 and AMC053 further supports the hypothesis that expression of *psbAI* is enhanced by decreasing light availability.

Differential expression of the *psbA* genes in *Synechococcus* may result from regulation at several levels. One such level of potential regulation is differential transcript stability. Since the *psbA-lacZ* transcripts were much less stable than native *psbA* transcripts, they were likely excluded from any posttranscriptional regulatory mechanism that might be associated with native transcript stability. Of the three *psbA-lacZ* constructs, only the *psbAI-lacZ* gene fusion on plasmid pAM129 was followed by the wild-type 3′ end of the gene. We do not know where transcription terminates for any of the *psbA-lacZ* gene fusions. Since all three of the *psbA-lacZ* fusion messages were relatively less stable than the respective *psbA* transcripts, differential mRNA stability cannot account for observed differences in β-galactosidase activity.

The viability of strains that contain all combinations of two selectively inactivated *psbA* genes demonstrates that both forms of D1 can function normally in the photosystem II reaction center (6). Assuming equal translation of *psbA* transcripts under normal conditions, the greater proportion of *psbAI* mRNA relative to that of *psbAII* and *psbAIII* in wild-type cells should be reflected in the relative amounts of the two forms of D1 in the thylakoid membrane, with form I predominating. Indeed, different ratios of form I to form II of D1 in the thylakoid membrane of wild-type cells have been observed on Western blots in our lab by using form-specific antisera raised against purified *psbA-lacZ* fusion proteins (20a). The form-specific antisera verifiably recognize the unique amino termini of the two forms of D1 and show that, in wild-type cells, both forms are present in the thylakoid membrane, albeit in different amounts.

Expression of *psbAI* and *psbAIII* account for the majority of *psbA* expression under our standard conditions. Even at 600 $\mu E \cdot m^{-2} \cdot s^{-1}$, when *psbAII* expression was at its observed maximum, this value was only 6% that of *psbAI* (at its observed minimum). Assuming unbiased incorporation of the two forms of D1 into the thylakoid membrane, the composition of the membrane would be directed by the contribution of each *psbA* gene product to the D1 pool at a given time. These data predict an increased proportion of form II to form I of D1 in the membranes of cells under conditions of high light. We have compared the relative amounts of both forms of D1 in thylakoid membranes isolated from wild-type cells along the same light intensity gradient reported here. As expected, Western blot analysis of thylakoid membranes from cells cultured under high light showed more form II and less form I of D1, compared with analysis of membranes from cells cultured under low light (20a). As PPFD decreased from 482 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 5 $\mu E \cdot m^{-2} \cdot s^{-1}$, the relative amount of form I increased 58% while the relative amount of form II decreased 60%. These data suggest that form II of D1 may provide a unique functional role in photosystem II under conditions of high light. The exact role for the two forms of D1 in *Synechococcus* remains to be determined.

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